Development of Extracellular matrix-nano Hydroxyapatite composite scaffold for Bone tissue engineering

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CERTIFICATE

This is to certify that the summer project report entitled, **"Development of Extracellular matrix-nano Hydroxyapatite composite scaffold for based bone tissue engineering** "submitted by **Mr. Nimal.T.R** in partial fulfillment of the requirements for the award of the Masters of Technology in Biotechnology and Medical Engineering with specialization in "Biotechnology" at National Institute of Technology, Rourkela is an authentic work carried out by him under my supervision and guidance.

To the best of my knowledge, the matter embodied in this thesis has not been submitted to any other University/ Institute for the award of any other Degree or Diploma.

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ABSTRACT

Adipose tissue has an extracellular matrix (ECM) which is enriched in the fibrillar protein collagen, elastin, basement membrane protein laminin, Glycosaminoglycans, and proteoglycans. All the components have very important role in tissue development and function, hence applicable in tissue engineering and regenerative medicine. We decellularised the adipose tissue using the combination of 1) Physical methods such as blending and freeze-thawing and the 2) chemical methods such as SDS. We found that the subsequent freeze dried decellularized adipose tissue results in highly porous scaffold of average pore size 257µm. We characterized the scaffold and found that it is suitable for soft tissue engineering applications but it is not suitable for bone tissue engineering because of its poor mechanical strength. To increase the mechanical strength, we prepared the composite of hydroxyapatite nanoparticle and ECM and characterized by FTIR and XRD. We prepared the Hydroxyapatite nanoparticle by wet chemical method and ECM-nano hydroxyapatite composite scaffold by the suspension method. The scaffolds fabricated possess a porous structure with an average pore size of 170µm and with an improved mechanical strength which is suitable for bone tissue engineering.

KEYWORDS: Extracellular matrix, Adipose derived stem cell, nanohydroxyapatite, bone tissue engineering

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1. INTRODUCTION:

1.1. Clinical Importance of Bone tissue engineering

There are over 1 million Bone Graft replacements happening in a year in order to obtain Unions on defective bone cases. India's fractures caused by arthritis and osteoporosis are alarmingly increasing [1]. According to census around Four hundred forty thousand Indians suffer from hip fractures, 26 hundred thousand suffer from osteoporosis and the same is expected to rise to 36 hundred thousand by fag end of 2013. Current diagnosis methodologies are majorly based on autologus bone crafts, autogeneous bone grafts or metal ceramics as an alternative approach [2-5].

To give an idea of these concepts, as explained here under.

- Autologous bone graft is a methodology in which a bone from another region of the patient body itself is used in the defective or fractured area and this has been the conventional method for decades. The commonly used bones for this methodology are the iliac crest that is taken in the form of trabecular bone and at times cortical bones are also used based on the cases [5, 7-8]. It is interesting to note that, although this case sounds to be successful in majority of times, it has its own limitations due to the limited amount of autograft that can be obtained and also due to donor site morbidity [2-5].

- Second methodology is the Allograft where a bone from another person's body is introduced into the recipient. However as it deals with two different bodies of different biological compositions, the rate of acceptance of new bone in the receipients body are much slower than the autologous method. In addition to this, there are also chances of immune rejection and pathogen transfer to the recipient body

thus causing a change in the immunological functioning of the recipient after transplantation [2-5].

- As an alternative to the above methodologies, metal ceramics is brought in picture [2]. Looking at the advantageous section of this technique is that they give immediate mechanical support and rigidity. However as a major consequence this methodology exhibits poor overall integration with the tissue and as a result can fail due to infection due to fatigue loading [2]. Also ceramics are of low tensile strength and cannot be used in locations which experience shear, high stress and bending.

Hence it is clearly seen that an adequate bone replacement is yet to be found and it is at the same time urgently needed for full recovery of the patients. A possible solution for these problems may be in TE.

1.2. Scaffolds used for tissue engineering:

A new therapeutic technology called Tissue engineering is more aimed at repair and regeneration of damaged tissues and organs. It is a detailed process of seeding of allogenic cells on scaffold (biomaterial) and the tissue construct is planted on the damaged area. The biggest advantage is the fact that injected cells remain in architecture of scaffolds and so do not have unnecessary diffusion of cells as in Stem cell therapy. The heart of this technology called scaffolds should be non-toxic, non-immunogenic, non-immunogenic, cytocompatible, biodegradable, hydrophilic and highly porous and it should have mechanical strength similar to that of target tissue or organ in totality to mimic native microenvironment. The various scaffolding materials used in this area can be broadly classified as polymers, ceramics etc [10].

The scaffolding materials using in tissue engineering can be divided in to polymers, ceramics and metallic as shown in fig 1

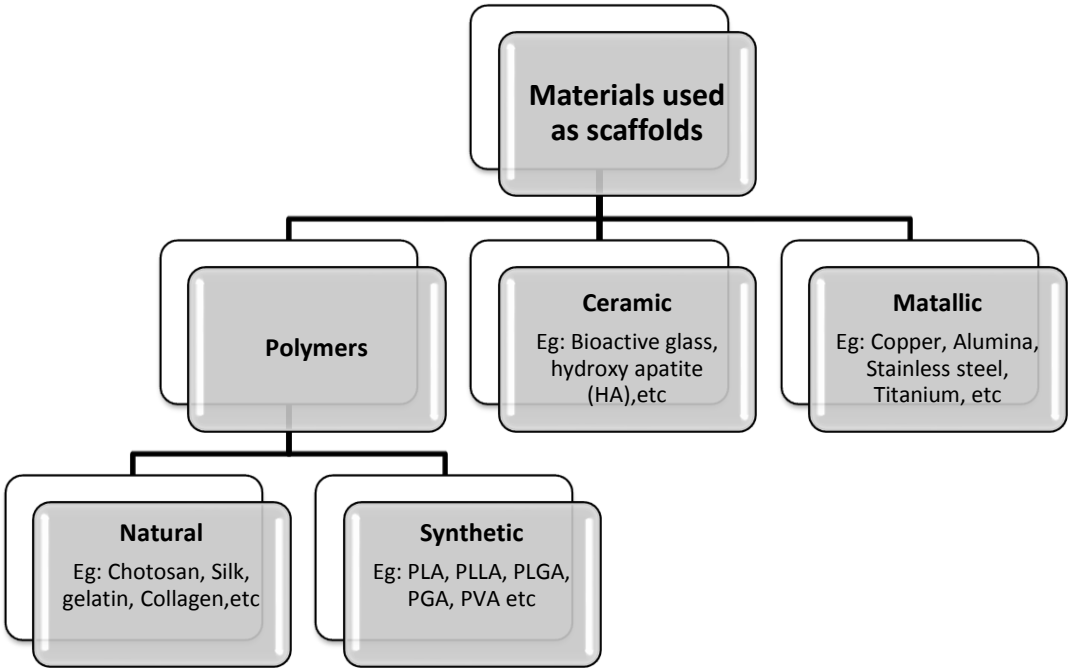


Figure 2: Classification of materials used for scaffold fabrication

Synthetic polymers, Ceramics (Except hydroxyapatite HA) and metallic scaffold cannot mimic body microenvironment in all aspects. But the combination of these with natural polymers like collagen, elastin, glycosaminoglycan (GAGs), etc can mimic native extra cellular matrix (ECM) in some extent. The usage of ECM as a scaffold material can mimic the native ECM completely with negligible immune rejection and they can support the seeded cells and direct the cell growth effectively.

2. LITERATURE REVIEW

2.1. Significance of ECM in tissue:

Extra cellular matrix is an acellular component present around the cells. Fibers like collagen and elastin, proteoglycan, glycoproteins contribute to ECM [11]. ECM gives mechanical support for the cells, helps in differentiation and development of cells in the tissue and also possesses a great role in signalling pathways. Each component of ECM possesses different functions, some of the components and their functions are tabulated below.

Table 5: Core proteins found in the extracellular matrix

Extracellular Protein	Function	Main Tissue Locations	ref
Collagen Type I	• Type I collagen found rope-like structures in tendon, sheet-like structures in skin, and in bone it is reinforced with nHA. • It gives tensile strength for the tissues.	bone, dermis, tendon, ligaments, cornea	12-14
Collagen Type II	• provide tensile strength and confers on cartilage the ability to resist shearing forces • supports chondrocyte adhesion and may influence the differentiated phenotype of these cells	cartilage, vitreous body, nucleus pulposus and inter-vertebral disc	12, 13
Collagen Type III	• essential for type I collagen fibrillogenesis during normal development of the cardiovascular system and other organs	Skin, vessel wall, reticular fibres of most tissues (lungs, liver, spleen, etc.)	15, 16
Collagen Type IV	• The assembled type IV collagen meshwork provides a scaffold for the assembly of other basement membrane components through	basement membranes	17, 18

	specific interactions with laminin, entactinlnidogen and heparan sulphate proteoglycan • this meshwork endows the basement membrane with a size-selective filtration property.		
Collagen Type V	• forms heterotypic fibrils with type I or types I and III collagens and help to provide mechanical strength for tissues.	bone, tendon, cornea, skin, blood vessels and the more compliant tissues, liver, lung and placenta.	[19, 16]
Collagen Type VI	• roles in tissue organization and remodeling	widespread: dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc	[20-22]
Collagen Type VII	• major component of anchoring fibrils • Anchor the basement membrane to the underlying stromal tissue.	epidermis and intestinal sub-mucosa	[23]
Collagen Type VIII	• serve as a molecular bridge between different types of matrix molecules • In different tissues this collagen type may serve different functions. Stabilization of membranes, angiogenesis, and interactions with other extracellular matrix molecules.	Descemet's membrane endothelial cells	[24, 25]
Collagen Type IX	• participates in the formation of, type II collagen fibrils • linking type II collagen molecules	cartilage, cornea , intervertebral	[16, 26, 27]

	• serve to bridge collagen fibrils with other matrix macromolecules	disc and vitreous humour	
Collagen Type X	• Involved in the process of mineralization, endochondral ossification, and is also proposed to play a role in angiogenesis.	Calcifying cartilage	[25, 28]
Collagen Type XI	• forms heterotypic fibrils with types II and IX collagens	Cartilage, inter-vertebral disc and vitreous body	[29]
Collagen Type XII	• modulate biomechanical properties of tissue	Skin, tendon, ligaments and cartilage	[16]
Collagen Type XIII	• transmembrane component of focal adhesion sites	epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs, liver, placenta, bone, and striated muscle	[16]
Collagen Type XIV	• found in region where high mechanical stresses are involved	dermis, tendon, vessel wall, placenta, lungs, liver and cartilage	[30]
Collagen Type XV	• Function to adhere basement membranes to underlying connective tissue stroma. Mouse studies have shown that collagen XV deficiency is associated with muscle and microvessel deterioration	fibroblasts, smooth muscle cells, kidney, pancreas	[16]
Collagen	• To maintain the integrity of the extracellular	Heart, kidney,	[31, 32]

Type XVI	matrix.	intestine, ovary, testis, eye, arterial walls and smooth muscles.	
Collagen Type XVII	• role in maintaining the linkage between the intracellular and the extracellular structural elements involved in epidermal adhesion	Hemidesmosomes of specialized epithelia and dermal–epidermal junctions	[16]
Collagen Type XVIII	• a potent antiangiogenic protein	Kidney, liver, lung	[33]
Collagen XIX	• To maintain the integrity of the extracellular matrix.	human rhabdomyosarcoma	[34]
Elastin	• provide elasticity and resilience to tissues • Elastin promotes cell adhesion and elastin peptides have been shown to be chemotactic.	Arteries, lungs, elastic ligaments, the skin, and the bladder, elastic cartilage.	[35]
Laminins (large glycoproteins)	• roles in development, differentiation and migration through their ability to interact with cells via cell-surface receptors, and with other basement membrane components such as type IV collagen, entactinlnidogen and heparan sulphate proteoglycan cell attachment and neurite outgrowth promotion.	basal lamina foundation for most cells and organs	[36]
Fibrillin-1 (glycoprotein)	• provide the scaffold on to which elastin is assembled to form elastic fibers • functional role in non-elastic tissues is unclear	Connective tissues.	[37]
Fibrillin-2	• Play a role in early elastogenesis.	Connective	[38]

(glycoprotein)		tissues.	
Fibromodulin (small chondroitin sulphatelder matan sulphate proteoglycan)	• to modulate collagen fibre formation • fibromodulin binds types I and II collagen fibrils in vitro and inhibits collagen fibril assembly.	Cartilage, tendon, skin, sclera and cornea	[39]
Link protein	• binds to both cartilage proteoglycan (aggrecan) and hyaluronan in cartilage extracellular matrix, thereby stabilizing their aggregation and producing supramolecular assemblies.	cartilage	[40]
Matrix Gla protein	• Act as an inhibitor of calcification in arteries.	cartilage and most visceral organs	[41]
MMP (matrix metalloprotei nases)	• cleaves collagens type I, II, III, VII, VIII and X, in addition to gelatine (non-triple helical collagen) ,proteoglycan, etc • Play a major role on cell behaviors such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defense.	Skin, basement membrane	[42]
Entactin/nido gen (sulphated glycoprotein)	• It associates specifically with both laminin (in a 1 : 1 molar ratio) and type IV collagen and is thought to play an important role in linking these two molecules together in the basement membrane	Basement membranes	[43]
Perlecan (large proteoglycan)	• It endows basement membranes with fixed negative electrostatic charge and is responsible, in part, for the charge-selective ultra-filtration properties of this extracellular	Basement membranes	[44]

	matrix. • It interacts with other basement membrane components such as laminin and collagen type IV, and serves as an attachment substrate for cells.		
Aggrecan(a large Chondroitin sulphate proteoglycan)	• to swell and hydrate the frame work of collagen fibrils in cartilage	Cartilage	[45]
Bamacan(chondroitin sulphate proteoglycan)	• stabilize the chromosomal scaffold at mitosis	basement membranes	[46]
Biglycan (small CS/DS proteoglycans)	• Biglycan interacts with collagen, • Mineralisation of bone. • Biglycan core protein binds to the growth factors BMP-4 and influences its bioactivity	bone ,cartilage and aorta	[47]
Cartilage matrix protein	• may act as a marker for post-mitotic chondrocytes	tracheal, nasal septal, xiphisternal, auricular and epiphyseal cartilage, but not in articular cartilage or extracts of the intervertebral disc.	[48]
Chondroadherin (leucme-rich glycoprotein)	• mediating cell attachment of chondrocytes to plastic, although it does not promote cell spreading,	Cartilage.	[49]

Dentin matrix phosphoprotein	• Play a role in mineralization.	Teeth, calvaria, preameloblasts.	[50, 51]
Fibronectin (glycoprotein)	• Fibronectin attaches cells to all matrices except type IV that involves laminin as the adhesive molecule • Cellular migration during development and wound healing, regulation of cell growth and differentiation, and haemostasis/thrombosis.	Basement membranes, cartilage, adipose tissue	[52]
Fibulin (glycoprotein)	• to interact with numerous extracellular matrix constituents including fibronectin, proteoglycans, laminins and tropoelastin	Basement membranes	[53]

2.2. **Significance of ECM as scaffolding material:**

ECM is a novel biomaterial for tissue engineering application, because it plays a major role in cellular growth, proliferation, cell attachment and movement of cells, etc. ECM scaffolds are highly compatible with tissue. The scaffolds even after sterilisation retain some growth factors, functional proteins and cytokines. Because of the component diversity in the ECM it helps in cell anchoring, cell metabolism also [54, 55]. following points describe the importance of ECM as scaffolding material in detail.

- ECM influences the cell phenotype and control the cell proliferation and cell differentiation also. Researchers found that ECM from Urinary Bladder Matrix (UBM) strongly affects smooth muscle cells phenotype, differentiation, etc [56].
- When ECM implanted in to body it start to remodel then degrading or resorbing like other scaffolds. During the remodelling the cells from neighbouring tissues will repopulate in to scaffold and cells like blood-borne and marrow-borne derived cells

migrate to the site of implantation and then they will start to differentiate in to a particular phenotype [57].

- ECM scaffolds possess can posses variety of mechanical characters because the mechanical properties depends of many variables like source of ECM (xenogenic [58] human, body location, etc), different processing methods (Decellularization methods [59] cross linking and sterilization methods [60, 61] both the tissue and scaffold should posses similar compliance and should have same mechanical property of target tissue otherwise it may leads to failure in vivo.
- The ECM Scaffolds shown resistance to infection after contamination with bacteria or gastrointestinal contents [62-64], after the transplantation of scaffold in to human body it will start degradation. During the biodegradation process small peptides (5 kDa to 16kDa) released from the fibers like collagen , these molecules mimic some peptides that inhibit the growth of gram positive and gram negative bacteria in in vitro [65].

  The preclinical studies show that ECM scaffolds shown resistance towards deliberate bacterial contamination [66, 67]. The clinical studies of ECM performed shown resistance towards spontaneous contamination [68-70].
- ECM Scaffolds also shows chemoattractant properties [62]. The components derived during the biodegradation process helps in the recruitment of cells to the remodelling site, and help in the tissue specific differentiation by mechanical and biochemical signals [70, 71]. In a in vivo study ECM shows an attractive property towards circulating bone marrow-derived cells and they will remain in the remodelled tissue [72, 74].
- ECM plays an important role in stem cell differentiation [75]. Many studies shows that decellularized ECM help in the stem cells differentiation and maintain the phenotype of the differentiated cell line in a tissue specific manner [54, 76, 77].

- The peptides released during the biodegradation on ECM components like collagen, elastin, laminin and fibronectin participate in mellaprotease matrix metalloprotease (MMP) expression, Cellular activity modulation and growth factor signalling, tumour vascularisation and angiogenesis [74, 78, 79].

Because of theses significance ECM scaffolds becoming popular in market some of them are tabulated in (Table 2)

Table 6: ECM products available in market

	ECM Product Name	Company	Source of ECM	Applications	Ref
1	Oasiss® Wound matrix & Oasiss® Ultra Tri-Layer Matrix	Healthpoint	Porcine small intestinal submucosa (SIS)	• Partial and full-thickness wounds • Pressure ulcers • Venous ulcers • Chronic vascular ulcers • Diabetic ulcers • Trauma wounds (abrasions, lacerations, second-degree burns, skin tears) • Draining wounds • Surgical wounds (donor sites/grafts, post-Mohs' surgery, post-laser surgery, podiatric, wound dehiscence)	80
2	TissueMend®	TEI Bioscience	fetal and neonatal bovine acellular dermal matrix	• Tendon repair surgery, including reinforcement of the rotator cuff, patella, achilles, biceps, quadriceps, or other tendons.	81
3	Xenform™	TEI Bioscience	fetal and neonatal bovine acellular dermal matrix	• to restore vaginal form and function after a pelvic floor procedure	81

4	Durepair ®	TEI Bioscience	fetal and neonatal bovine acellular dermal matrix	• repair of the dura mater during neurosurgical procedures	[81]
5	PriMatrix AG ™	TEI Bioscience	fetal and neonatal bovine acellular dermal matrix	• Skin ulcers • Trauma wounds • Second degree burns • Full thickness wounds • Partial thickness wounds • Surgical wounds • Post-Mohs surgery • Tunneled wounds	[81]
6	SurgiMend PRS ™	TEI Bioscience	fetal and neonatal bovine acellular dermal matrix	• Designed specifically for plastic and reconstructive surgery and made of pure collagen that provides sound support for rapid healing – without inflammation.	[81]
7	MemoDerm	MMI Foot and Ankle	Human dermis	• Rotator Cuff • Anterior Shoulder Capsule • Flex/Extensor Tendon • Ulnar Collateral Ligament • Achilles Tendon • Lateral Ankle Complex • Chronic Diabetic foot Ulcer	[82]
8	DermaMatrix	DePuy Synthes	Human dermis	• Facial—soft tissue defects • nasal reconstruction • septal perforation • parotidectomy • Intraoral—cleft palate repair, • oral resurfacing • Vestibuloplasty • Radial forearm free flap repair • Breast reconstruction postmastectomy	[83]

				• Abdominal wall repair.	
9	AlloDerm	LifeCell	Human dermis	act repair in challenging hernia east reconstruction postmastectomy	[84]
10	GraftJacket ®	Wright Medical Technology	Human dermis	• Replacement of damaged or inadequate integumental tissue.	[85]
11	MATRI DERM ®	Ideal ™ Medical Solutions	Bovine dermis	• burns or other kind of injuries to the skin	[86]
12	MatriStem™ Wound Care Matrix	Medline	Porcine urinary bladder Matrix	• Chronic and acute wounds (partial and full-thickness, draining, trauma, surgical) • Ulcers (diabetic, venous, pressure, and chronic vascular).	[87]
13	EZ-DERM ®	WOUNDSOURCE	Porcine dermis	• Partial-thickness skin loss, donor sites, skin ulcerations and abrasions • Temporary covering for full-thickness skin loss, Toxic Epidermal Necrolysis (TEN) and meshed autograft protection.	[88]
14	Biodesign® (Surgisis®)	Cook Medicals	Porcine small intestine submucosa (SIS)	• Hernia Repair • Fistula Repair • Plastic & Reconstructive/ENT • Staple Line Reinforcement • Peyronie's Repair • Continence Restoration • Dural Repair • Pelvic Floor Repair	[89]
15	Permacol™	COVIDIEN	Porcine dermis	• complex hernia repairs • abdominal wall reconstruction	[90]
16	CollaMend*	BARD	Porcine dermis	• hernia	[91]

	Implant	DAVOL LNC		• Abdominal wall repair.	
17	XenMatrix™ Surgical Graft	BARD DAVOL LNC	Porcine dermis	• hernia • Abdominal wall repair.	[92]
18	BIOPAD ®	Argentum medical LLC	Equine flexor tendon	• ulcer and skin lesion to help wound closure	[93]
19	Strattice™ Reconstructive Tissue Matrix	Life Cell	Porcine dermis	• Abdominal wall reconstruction • Breast reconstruction postmastectomy • Breast plastic surgery	[94]
20	Endoform™ Dermal Template	Mesynthes	Propria-submucosa layers of ovine forestomach	• partial and full-thickness wounds • pressure ulcers • venous ulcers • diabetic ulcers • chronic vascular ulcers • tunnelled/undermined wounds • surgical wounds (donor sites/grafts, post-Moh's, post-laser, podiatric, wound dehiscence) • trauma wounds (abrasions, lacerations, second-degree burns, and skin tears) • draining wounds	[95]
21	Veritas® Collagen Matrix	Synovis	Bovine pericardium	• hernia repairs • plastic and reconstructive surgeries, • Pelvic floor procedures.	[96]
22	SureDerm™ Acellular Dermal Graft	HANS Biomed	Human dermis	• Soft tissue coverage • Hypertrophic scar revision • Facial soft tissue repair	[97]

				• Skin defect, Burn wound allograft • Protective covering for nerves, tendons	
23	AlloMax™ Surgical Graft	BARD DAVOL LNC	Human dermis	• hernia • Abdominal wall repair.	[98]
24	NeoForm™	Mentor Corporation	Human dermis	• Breast Reconstruction	[99]
25	FlexHD® Acellular Hydrated Dermis	Ethicon	Human dermis	• Breast Reconstruction	[100]
26	CuffPatch™	BioArthro	human amniotic tissue	• Rotator cuff repair.	[101]
27	Tendon Wrap™	BioArthro	human amniotic tissue	• Protection of tendons after repair.	[102]
28	Durasis®	Cook Medicals	Porcine small intestinal submucosa (SIS)	• for repairing dura mater	[103]
29	Stratasis®	Cook Medicals	Porcine small intestinal submucosa (SIS)	• Treat stress urinary incontinence resulting from urethral hypermobility (Type II) and/or intrinsic sphincter deficiency.	[104]
30	OrthADAPT ™	Pegasus Biologicals	Horse pericardium	• Posterior Tibial Tendon Repair	[105]
31	BioSet®	PENTA BIOMEDICAL	Human bone	• Bone regeneration	[106]
32	Sterling®	PENTA BIOMEDICAL	bovine bone	• Bone regeneration	[107]
33	Restore™	DePuy	Porcine small	• To reinforce soft tissue where	[10

			intestinal submucosa (SIS)	weakness exists, specifically for areas of soft tissue repaired by sutures or suture anchors, including the rotator cuff, patella, achilles, biceps, quadriceps, and other tendons	[8]
34	Dura-Guard®	Synovis Surgical	Bovine pericardium	• Tumor resection • Trauma • Congenital disorders • Cerebrovascular disorders	[109]
35	Vascu-Guard®	Synovis Surgical	Bovine pericardium	• Carotid endarterectomy • Profundaplasty • Arteriovenous access revisions • Femoral, iliac, renal and tibial patching	[110]
36	Peri-Guard®	Synovis Surgical	Bovine pericardium	• Pericardial closure • Intracardiac repair • Great vessel repair • Septal and annulus repair • Soft tissue repair (abdominal wall defect, thoracic wall defect, hernia.)	[111]
37	Zimmer Collagen Patch®	Tissue Science Laboratories	Porcine dermis	• Reinforcement of large tendon tears at the repair site	[112]

2.3. Preparation of ECM- Decellularization:

Decellularization is used to remove the immunogenic cellular components from the tissue without hampering molecular structure and orientation of extracellular matrix components.

Differ physical, chemical and enzymatic methods are available for the Decellularization. Use of combination of different Decellularization methods showed better result than using individual methods.

An Ideal Decellularization should not affect the structure and properties of extracellular components like collagen, elastin, glycosaminoglycan etc. But in reality all the Decellularization protocols shows some drawbacks.

Freeze-thaw cycle is a common physical Decellularization method; the ice crystals formed during the process disrupt the cell membrane but as well as they can fracture ECM also. Alkali and acid treatment help to solubilise cytoplasmic components and degrade nucleic acids but the digestion cause the loss of GAGs.

Detergents are effective decellularising agents because they have a great role in plasma membrane disruption. Ionic, non ionic and Zwitter ionic detergents can be used for the Decellularization but their mode of action will be different. Non ionic detergents like Triton X100 Disrupts lipid–lipid and lipid–protein interactions but GAGs content after the treatment will decrease. Ionic detergents like Sodium dodecyl sulfate (SDS) Solubilise cytoplasmic and nuclear cellular membranes but they denature collagen and other proteins and also decrease the GAGs concentration in the ECM. Zwitterionic detergents like CHAPS show the properties of both ionic and non ionic detergents. Cell lysis can be archived by osmotic shock by a hypertonic solution like highly concentrated sodium chloride solution but the removal of cellular component needs further processing.

Enzymatic treatments also can be used for Decellularization. Trypsin is an enzyme which commonly used for Decellularization process but prolonged exposures of the trypsin can loss of different ECM components. Immunogenic components can be removed with the help of endonucleases and exonucleases but the sample should be washed thoroughly after treatment because the removal of enzymes is very difficult it can leads to immune responses.

Table 7: Decellularisation method for different tissues

Sl No	Tissue	Decellularization methods	Ref
1	myocardial tissue	1% (wt/vol) sodium dodecyl sulfate (SDS) for 4-5 days 1% (vol/vol) Triton X-10 0 for 30 min stir overnight in deionized water to ensure removal of detergents	113
2	urinary bladders	Isolation of UBM (Soak in 1N Saline or water (Urothelial layer removal) and Mechanically delamination of The tunica serosa, tunica muscularis externa, tunica submucosa, and most of the muscularis mucosa) 0.1% (v/v) peracetic acid, 4% (v/v) ethanol, and 96% (v/v) deionized water for 2 hours. phosphate-buffered saline (pH- 7.4) wash twice (15min each) deionized water. Wash twice (15 min each)	114, 115
3	Adipose tissue	Freeze-thaw 3 times Incubate for 16 hours (overnight) in Enzymatic Digestion Solution 1 Polar solvent extraction in 99% isopropanol Rinse 3 times (30 minutes each) in Sorenson's Phosphate Buffer Incubate for 6 hours in Enzymatic Digestion Solution 1 Rinse 3 times (30 minutes each) in SPB Incubate for 16 hours (overnight) in Enzymatic Digestion Solution 2 Rinse 3 times (30 minutes each) in SPB Polar solvent extraction in 99% isopropanol (8 hours) Rinse 3 times (30 minutes each) in SPB	54, 116
4	Liver	portal perfusion with SDS	117
5	Adipose Tissue	Homogenization of tissue: water mixture (2:1) for 3 min with pulsation Centrifugation for oil separation (3000 rpm for 5 min) Distilled water wash for several times Viscous suspension cast in a shallow mold Freeze drying 0.5% SDS wash of scaffold for 1hr at room temperature Wash properly with distilled water for 24hrs at 4^{o}C	55

6	small intestinal submucosa (SIS)	Mechanical Delamination (to remove the luminal portions of the tunica mucosa, including the majority of the lamina propria, and the tunica muscularis externa and serosa) 0.1% (v/v) peracetic acid (σ), 4% (v/v) ethanol, and 96% (v/v) deionized water for 2 h washed twice for 15 min with PBS (pH = 7.4) and twice for 15 min with deionized water	118-120,
7	Liver	Freezing of liver lobes(-80°C) Cut in to slices of 5mm Treat with enzymatic and detergent solutions DI water wash for 30 min (3 times) Treat with 0.02% trypsin/0.05% EDTA at 37°C for 1 h Rinse in DI water Treat with 3% Triton X-100 for 1hr Place the slices in 4% sodium deoxycholic acid for 1 h Rinse with DI water	118
8	Adipose tissue	Thawing of tissue Mechanical massaging 0.02% trypsin/0.05% ethylenediaminetetraacetic acid Rinse in distilled deionized water Mechanical massaging 3% triton X-100 Rinse in distilled deionized water 4% sodium deoxycholate Rinse in distilled deionized water 4% ethanol/0.1% peracetic acid Rinse in PBS and distilled deionized water Lyophilization 100% n-propanol Rinse in distilled deionized water Lyophilization	121
9	Adipose tissue	1% sodium dodecyl sulfate (SDS) in deionized (DI) water or 2.5 mM sodium deoxycholate in 1X PBS (48h) 2.5 mM sodium deoxycholate in 1XPBS supplemented with 500 U of porcine lipase and 500 U of porcine colipase (24-48h) Lyophilisation and milling	122

10	Adipose tissue	tissue/water mixture was homogenized at 12,000 rpm for 5 min Centrifuged at 3000 rpm for 5 min and the upper layer containing oil components were discarded. Repeated above steps several times Freeze drying and milling	123, 124
11	Adipose tissue	homogenized at 12,000 rpm for 5 min at room temperature Centrifugation and oil removal 1M hyperosmolar solution of NaCl (diluted 1:1) for 2 h at 37°C Centrifugation and oil removal Rinsing with distilled water for 24 h at 4°C 0.5% sodium dodecyl sulfate (SDS) for 1 h at room temperature Centrifugation and water wash 0.2% DNAase (2000U) and 200 µg/mL RNAase for 1 h at 37°C Centrifugation and water wash.	123
12	Skeletal muscle	Chloroform/methanol (2:1 v/v) extraction of lipid Graded series of alcohol (100,90,70,50,0) 30min each 0.2% Trypsin/0.2% EDTA at 37 °C (2h) Deionized water, 2XPBS (30min each) 2% sodium deoxycholate (5h) Deionized water, 2X PBS (30min each) 2% sodium deoxycholate for 14-16h 1% Triton X-100 for 1h Deionized water (30min) 0.1% (w/v) peracetic acid/4% ethanol (v/v) (2h) 1 X PBS, 30 min each twice Deionized water, 30 min each twice.	125
13	Dermis	Mechanical delamination (The subcutaneous fat, connective tissue, and epidermis were removal) 1 cycle freeze thaw (-80°C - 37°C) 0.25% trypsin for 6h three 15 min washes of deionized water 70% ethanol for 10 h 3% H2 O2 for 15 min two 15 min washes of deionized water 1% Triton X-10 0 in 0.26% EDTA/0.69% Tris for 6 h with a fresh change for an additional 16 h	126

		three 15 min washes of deionized water 0.1% peracetic acid/4% ethanol for 2 h two 15 min washes of PBS two 15 min washes of deionized water.	
14	central nervous system (optic nerve, spinal cord, and brain)	frozen (>16 h at 80 °C), thawed separated all non CNS Tissue cut into lengths (<3 cm) Agitations in following reagents : Agitation speed was 20 0 rpm for optic nerve and spinal cord tissues or 120 rpm for brain tissue with the exception of the initial step at 60 rpm. water (type I reagent water per ASTM D1193; 16 h at 4 °C; 60 rpm) 0.02% trypsin/0.05% EDTA (60 min at 37 °C) 3.0% Triton X-100 (60 min) 1.0 M sucrose (15 min) water (15 min), 4.0% deoxycholate (60 min) 0.1% peracetic acid in 4.0% ethanol (120 min) PBS (15 min) water (15 min) (2 times) PBS (15 min) Lyophilization.	127
15	Cartilage	The suspension of cartilage fragments was spun in centrifuge for 5 min at 2000 rpm Removed the deposit New suspension recentrifuged for 5 min at 7000 rpm incubated in 1% TritonX-100 in hypotonic Tris HCl with gentle agitation for 12 h at 4 °C 12 h in 50 U/mL deoxyribonuclease I and 1 U/mL ribonuclease A in 10 mM Tris HCl, pH 7.5 washed in PBS intensively	128
16	Tracheal Tissue	1 hour in 0.25% Trypsin/0.05% ethylenediaminetetraacetic acid at 37°C 48 hours in deionized water at 4°C 48 hours in 3% Triton X-100 at 4°C rinsed heavily Lyophilisation	129

17	pericardial tissue	40 ml of sterilized and filtered PIPES buffered saline solution (PSS, 30 mM, pH 7.4, 0.9% NaCl) containing 0.4 ml of tridecyl alcohol ethoxylate (1% v/v) and treated at 4 °C for 48 h under orbital stirring (20 rpm). The solution containing the surfactant was changed after 24 h and after this period of time, the samples were washed with PSS	13

2.4. ECM Scaffold Crosslinking and sterilization

- *Crosslinking of ECM Scaffolds:*

Cross linking methods used to improve different parameters of the scaffold like mechanical strength,biodegradability, thermal stability etc. ECM Crossliniking offers following advantages

- Increase ECM strength and achieve consistent mechanical properties
- To improve compressive stiffness
- To decrease biodegradability
- To inhibit recognition of surface epitopes by host that is to reduce antigenicity
- To inhibit cellular infiltration to the scaffold

Crosslinking can be achieved by different methods like Chemical crosslinking and Biophysical methods. Chemicals like Carbodiimides (eg: 1-Ethyl-3-3-dimethyl aminopropyl carbo-diimide EDAC), N-hydroxy sucinimide, Glutaraldehyde GTA, Hexamethylene diisocyanate can be used for crosslinking. EDAC form zero length cross links, which are chemically identical to formed by dehydrothermal method (a biophysical method). GTA links form long polymer chains, so EDAC can crosslink fiber with a distance less than 1nm only,

but GTA can crosslink fibers with distance. GTA crosslinked have ability to support 6.4times as many cells as the standard dehydrothermal (biophisical method).

Chemical crosslinking can cause damages to ECM, the drawbacks are as follows

- Chemical cross linking changes ultra structure of ECM scaffold however it cause minimal changes to molecular composition
- It may alter presentation and confirmation of ligands on the ECM surface thus altering ligand-receptor interactions
- GTA and Hexamethylene diisocyanate reduce the ability of cells to interact with treated material and to cause cutaneous sensitization and to lead to calcification
- May inhibit cell migration and vascular regeneration.
- Residual crosslinker may produce inflammatory response

Because of above mentioned drawbacks researchers using biophysical methods like dehydrothermal treatment, UV irradiation etc. In dehydrothermal treatment scaffold is treated at high temperature in vacuum for 12-24 hrs. During the dehydrothermal treatment water removed will create some links between strands of collagen, elastic fibers etc.

- *Sterilization of of ECM Scaffolds:*

Sterilization of scaffold is very important to reduce risk of disease and required for FDA clearance. Peracetic acid, ethylene oxide treatment, gamma irradiation are the commonly used sterilization methods for ECM.

Peracetic acid PAA: (0.1-0.15%): During the treatment the PAA enter in to microbial cells and oxidize microbial enzymes and it also kills human cells also so it can be used for

Decellularization. But PAA oxidize the matrix and reduce structural integrity of collagen fiber oxidative destruction of GAGs fracture them, impairs their ability to interact with growth factors and subsequently increases the susceptibility of growth factors to denature.

Ethylene oxide gas: is widely used sterilization method for ECM products because other available methods have deleterious effects. The gas causes the alkylation of matrix and helps to makes the scaffold free from microorganism including bacteria. But alkylate matrix proteins, rendering them inactive and undetectable. PAA and Ethylene oxide treatment ensure viral safety and sterility. Ethylene dichloride (EDC) also a sterilizing agent but during treatment it preserve collagen structure in tissue.

Chemical used for sterilization can trapped inside scaffold and may cause inflammatory response in this scenario gamma irradiation have great importance. Different range of Gamma irradiation can be used for sterilization. At low dosage of gamma irradiation (<15KGy), the strength and modulus of scaffold increased but mechanical property decreased above 15KGy in a dose-dependent manner. The change is due to the increase in collagen cross-linking due to the low dose of irradiation that levels off after approximately 5KGy, along with collagen chain scission that continues to increase with irradiation dose.

2.5. Nano Hydroxy apatite

Nanosized hydroxyapatite (HA) is the main component of mineral bone. The bone remodelling comprises of a coupled resorptive-formative process where all the living bones undergoes this process constantly. The bone cells osteoblast and osteoclast brings about simultaneous bone removal and replacement process through their respective activities.

HA possesses similarity with the hard tissues of the body with its exceptional biocompatibility and bioactivity with bone cells and tissues. These calcium phosphate biomaterials have been widely used in different forms such as powders, granules, dense and porous blocks and various composites in the clinical field. The main mineral part of calcified tissues is formed by these calcium phosphate materials only. But this calcium phosphate present in the form of nano meter-sized needle-like crystals of 5-20 nm width and 60 nm length approximately in the bone, with a poorly crystallized non-stoichiometric apatite phase containing various ions like $(CO_3)^{2-}$, Na^+ and F^- in a collagen fiber matrix.

Compared to other conventional ceramic formulation. HA properties such as surface grain, wettability, pore size, etc, can be varied to control protein interactions. So that one can modulate the adhesion of osteoblast and its long term functionality. Webster et al [32-134] found that by controlling this properties osteoblast functions such as proliferation, alkaline phosphatase synthesis and calcium containing mineral deposition can be enhanced. These properties not only promote increased selective vitronectin adsorption and also affect conformations which can enhance osteoblast functions.

2.6. Bio-orthopedic properties of nanoHA

The removal of infected bone tissue or bone tumours leads to the need of bone substitutes for a individual. The first desirable form of bone substitutes is autologous bone. However, autografts have their own disadvantages like availability and morbidity at the donor site. Second desirable form is an allograft, but there is also possibility of host immune response and disease (i.e. human immunodeficiency virus (HIV) or hepatitis B) transmissions to the recipient [135]. Hence the advantages of Bone graft substitutes over both autografts and allografts bring about the attraction in recent years [136]. Bone

substitutes is simply a biomaterial having properties such as biomimetic poor crytallline, carbonate substituted apaptite with good mechanical strength to mimic natiural bone [137]. HAp artificial bones showed satisfying repair function as in the of clinical field over the past years [138–140]. Inspite of their success they possess some weak-nesses, such as weak intensity and slow degradation. Current research has been in progress to improve the properties of HA to meet both biological properties and the mechanical properties of the natural bone [141].

Recently HAp coating on scratched areas of a human tooth and HAp disks by the immersion method in a HAp colloidal solution were reported [142]. The surface morphologies of the damaged surfaces were remarkably recovered after 3 months of immersion of HA. The cell study with mouse calvaria-derived pre-osteoblast cells (MC3T3-E1) showed that HA layers could be suitable for maxillofacial and orthopedic applications. Another interesting result showe by the same research group that nanoscale HA powders (mean particle size-200 nm) were used to regenerate the enamel layers of damaged teeth [143]. A nanoscale HAp powder suspension in deionized water was immersed in an artificially scratched tooth for a period of 1–3 months. After 3 months, the scratched surface was finally examined where there is increase in the roughness , newly generated HA layer which was very similar to that of innate layer. Ca^{2+} and $(PO_3)^{4-}$ ions from the HA were precipitated on the tooth to produce cement pastes on the enamel surface resulting in the formation of new HA layer. This nanoscale HAp powder solution might be used to heal decayed teeth as well as to develop tooth-whitening materials. A synthetic bone in sinus bone grafting called SB-1™ consists of synthetic HAp with 500–1400 l m particle size and chemical composition similar to that of inorganic part of human bones. It was found through an in-vivo study with rabbit that SB-1™ was exceptionally good in terms of bonding with host bones and also inducing the formation

of new bone as well as integration with new bone in comparison with other allografts and low HA bone grafts.

The osteoconductive properties of nano-HA material and its potential application were evaluated by Zhu et al. [135] on the basis of the cross-sectional area filled with mineralized bone by HA scaffolds. And the study showed that highly porous and interconnected HA scaffolds synthesized were potentially suitable for the orthopedic application

Table 8: Current status of Research on ECM based tissue engineering

1	ECM source	Decellularization method	Solvent parameters	ECM Treatment	Result	Ref
1	1.porcine small intestinal submucosa(SIS) 2.porcine urinary bladder submucosa(UBS) 3.porcine urinary bladder matrix(UBM) 4.a composite of 5.UBS+UBM canine stomach submucosa	Mechanical delamination	--	0.1%Peracetic acid treatment with 4% ethanol (disinfection) & Vacuum drying Multilaminate extracellular matrix devices by mechanical pressing.	Different 4 layer and 2 layer laminated devices of UBS, UBM and SIS shown different mechanical property. So according to the strength of target soft tissue we can produce scaffolds by lamination method using ECM.	142
2	Pig UBM Pig SIS Pig Liver ECM(LECM)	UBM & SIS: mechanical delamination LECM: Trypsin, EDTA, Triton X-	--	Peracetic acid treatment & Crosslinking : either carbodiimide ethylene oxide gas sterilization	The structural and morphological characters of scaffold depend on many factors like source of tissue or organ. Chemical cross linking affect the surface characteristics of ECM scaffolds.	143

		100, sodium deoxycholic acid, peracetic acid, ethanol treatment				
3	Acellular PCL/ECM composite PCL-Cartilaginous ECM Fibers (Electrospinning)	Freeze thawing	PCL (14 wt %) in a of chloroform to methanol (5:1 volume ratio). flow rate of 18 mL/h Voltage 25.5 kV	After Decellularization, - fitted into Cassettes Sterilization: ethylene oxide gas for 14 h	ECM can stimulate stem cell differentiation. ECM-PCL composite scaffold support chondrogenic differentiation.	144
4	Human bone marrow mesenchymal stem cells (MSCs), normal human dermal fibroblasts (NHDF, and) normal human articular chondrocytes (NHAC) cultured on PLGA mesh and decellularized the construct.	Freeze thawing (6 times -80°C to room temperature) and Ammonia treatment	--	PLGA mesh solubilisation by trisodium phosphate freeze-dried	The ECM scaffolds shown excellent biocompatibility when implanted to animal model. By culturing method they developed allogenic scaffolds..	145
5	Canine skeletal muscle ECM (M-ECM) porcine (SIS)	M-ECM: Chloroform/ methanol, Trypsin, EDTA, sodium deoxycholate, Triton X-100, peracetic	--	Lyophilized and sterilized with ethylene oxide gas	The M-ECM shows effects on myogenic cells in vitro and help in positive remodelling in rodent muscle defect model. tissue specific M-ECM was not showing any significant advantage over homologous SIS scaffolds	146

		acid				
6	porcine optic nerve, spin al cord, and brain ECM	Trypsin, EDTA, Triton X-100, sucrose, Deoxycholate, peracetic acid treatments	--	lyophilized	The ECM scaffolds developed influences proliferation; differentiation and migration of PC12 cell lin in vitro. Central nerve system CNS and non-CNS acellular matrix is the best scaffold for neural tissue remodelling and CNS repair	147
7	mineralized (ECM) and electrospun poly(e - caprolactone) (PCL) microfiber scaffolds	freeze and thawing (3 cycles-LN2 -37°C)	PCL (14 wt %) in a of chloroform to methanol (5:1 volume ratio). flow rate of 18 mL/h Voltage 25.5 kV	Sterilization: ethylene oxide gas for 14 h	The scaffold developed stimulates osteogenic differentiation of MSCs in vitro.	148
8	Bovine articular cartilage ECM	TritonX-100, Tris HCl, DNAase I , RNAase A.		lyophilized scaffolds cross-linked by genipin solution sterilized by to 20 kGy ^{60} Co irradiation	The scaffold combined with BMSCs which are chondrogenic-induced developed in to functional tissue engineering cartilage in 4 weeks of culturing. The BMSC start to secrete cartilage ECM that will enhance the biomechanical properties.	149
9	Human cartilage ECM	Pulverization, differential centrifugation, TritonX, DNAase I , RNAase A		Cross-linking: DHT & carbodiimide. Sterilization by ^{60}Co gamma radiation.	The scaffold developed retains most of the bio molecules of ECM and possess good biocompatibility. The novel scaffold retains most of the cartilage. The scaffolds also help in the proliferation and differentiation of BMSCs in to chondrogenic cell lines.	150
10	porcine UBM	Peracetic acid (PAA)		Mechanical pressing (plying)	The plying method is a best method to develop multi layered UBM. And can developed scaffold with different mechanical property.	151
11	Porcine UBM	Washing, delamination, peracetic acid/ ethanol treatment-	--	vacuum drying ethylene oxide sterilization	idea of applying ECMs with an SA better than their intended imperfection did lead to more efficient TBC and C values in comparison to conventional urological TE.	152
12	Porcine (UBM), and	UBM: delaminatio	--	carbodiimide crosslinking and	The ECM scaffold developed applied for tracheal regeneration successfully	153

	decellularized tracheal matrix (DTM)	n, peracetic acid/ ethanol treatment- DTM: Trypsin, ethylenediaminetetraacetic acid, Triton X-100		ethylene oxide.sterilization.		
13	Porcine (UBM), and (SIS)	delaminatio n, peracetic acid/ ethanol treatment-	--	vacuum pressing, lyophilised and sterilized with ethylene oxide	Multi laminated ECM membranes showed high elongation ability. properties.	154
14	Porcine (UBM)	delaminatio n, peracetic acid/ ethanol treatment,	---	--	The ECM scaffold developed applied for default mechanisms of esophageal repair successfully	155
15	porcine chondrocytes -derived extracellular matrix PGA Scaffold as control	Freeze thawing (3 cycles of -20℃ to room temperature) at 12 h intervals.	--	Freeze drying	The scaffolds developed from ECM secreted by cultured cells provide a efficient environment for cartiliage tissue engineering in vitro.	156
16	human adipose tissue ECM	Homogeniz ation, NaCl, SDS DNAase & RNAase treatments		Freeze drying	The decellularized method was effective and preserved the scaffold architecture. Elastic property of ECM was maintained even after Decellularization. The scaffolds also promote host cell infiltration in vivo.	157
17	Porcine SIS	PAA Freeze drying		ethylene oxide sterilization	Even after the treatment of Decellularization and sterilization retained Fibronectin, FGF-2 and glycosaminoglycans in scaffold. But VEGF content reduced dramatically	158

18	Porcine SIS ECM	PAA disinfection of SIS Freeze drying		ethylene oxide sterilization	The invitro bioactivity of processed SIS was examined on murine fibroblasts and PC12 cells after subjecting them to the effect of peracetic acid disinfection, lyophilisation and ethylene oxide sterilization. The post-sterilization SIS was specifically analyzed for its ability to enhance fibroblast attachment, stimulation of PC12 cell differentiation and upregulation of fibroblast VEGF secretion. The result showed increased attachment of fibroblast to sterilized SIS, retention of viability and increased secretion of VEGF to a two-fold level upon interaction with SIS matrix components. The PC12 cells were also observed to exhibit increase in neurite outgrowth as compared to controls due to stimulation by SIS matrix proteins. The scaffold can also be prepared for human use with significant bioactivity.	159
19	Porcine (SIS)	delaminatio n, peracetic acid/ ethanol treatment,			The use of xenogenic small intestine submucosa for the repair of the anterior crucitate ligament in goat model is a promising example of resorbable bio-scaffold development.	160
20	Porcine (UBM)	delaminatio n, peracetic acid/ ethanol treatment,		Sterilization: 2.0 M rad γ-irradiation	Constructive remodeling of myocardial tissue for the replacement of excisional defects was facilitated by using UBM as replacement scaffold.	161
21	human adipose tissue ECM	homogenize d, centrifuged, freeze-dried, and ground to powders by milling		Sterilization by ethylene oxide (EO) gas	In a particular in vivo study, the use of human ECM powder proved highly effective in promoting attachment and proliferation of cells in a three-dimensional (3D) cell culture. In this study, human ECM solutions containing adipose-derived stem cells (hASCs) were subcutaneously injected into nude mice. Eight weeks later, the growth of a large number of blood vessels and adipogenesis accumulating intracellular small lipid droplets were observed in the newly formed tissue. To summarize, the grafts showed a well patterned construct of adipose tissues without any signs of cystic spaces, tissue necrosis or fibrosis.	162
22	(UBM) and (SIS)	delaminatio n, peracetic acid/ ethanol	--	Sterilization by ethylene oxide (EO) gas	The success in Vascular tissue engineering has been shown to increase due to the use of ECM which promotes growth, proliferation, anchorage and migration of cells derived	163

		treatment,			from human vasculature. The architecture and other features of the scaffold also control the rate of adhesion and proliferation of cells. The phenotype of smooth muscles and their proliferation-differentiation mechanism control is observed to be influenced by UBM scaffold containing a rigid basal membrane. .	
23	Human Umbilical Vein Endothelial cells (HUVEC) ECM	EDTA/PBS will help to detach the cells and ECM will remain in culture dish			The attachment and proliferation of heterologous HDMECs and dermal fibroblasts on mixed matrix and HUVECs ECM proved successful. The lingering after effects of dispase were found to be neutralized by the conditioned medium from HUVECs which could also be used for tissue culture of epidermal keratinocytes, HDMECs and dermal fibroblasts whose extracellular secretions are common.	164
24	UBM or expanded polytetrafluoroethlyene (ePTFE)	Mechanical delaminatio n, hypotonic cell lysis, disinfection by peracetic acid (0.1%),.		Lyophilization, vacuum pressing,	Biodegradation of UBM when implanted as an LV-free wall infarction patch was accompanied by the appearance of a fibrocellular tissue including contractile cells. Hence UBM was proved superior against synthetic material for cardiac patching and helps in myocardial replacement within 3 months	165
25	Porcine UBM	delaminatio n, peracetic acid/ ethanol treatment,			The temporal and spatial architecture of innervations during the in vivo modeling of ECM scaffold used for the reconstruction of the esophagus and muscular body wall was demonstrated by the results of this study. A more detailed study in this prospect is required to be done.	166
26	Human Adipose tissue ECM	Freeze-thaw(3 times enzymatic digestion Polar solvent extraction.		A chemical drying method used for fabrication	This tissue source was found to be an excellent source for the isolation of human ECM in large quantities which also includes basement membrane components. Various methods were developed for the extraction of large-volume intact ECM scaffolds with a preserved three-dimensional architecture. An inductive microenvironment for the adipogenic differentiation of human ASC was provided by decellularized adipose tissue. DAT scaffolds, as compared to control monolayer or cell aggregate cultures, showed a higher expression of adipogenic markers when analyzed using gene expression studies.	167

27	Human Adipose tissue ECM	delaminatio n, peracetic acid/ ethanol treatment,		Air drying of ECM	The use of DAT-based microcarriers as injectable adipose tissue specific scaffolds for a dynamic culture system of ASC adipogenesis was investigated. The results positively showed that the microcarriers prepared by dissolving DAT provided a natural substrate for induction of adipogenic differentiation of human ASCs in vitro, which confirms our earlier work with intact DAT scaffolds. Gene expression studies also indicated that DAT microcarriers induced the expression of PPAR g C/EBP a and LPL without having to provide adipogenic differentiation factors exogenously.	168
28	Human Adipose tissue ECM	Freeze-thaw(3 times enzymatic digestion Polar solvent extraction.		sterilized by ethylene oxide gas	Decellularization and fabrication of human adipose tissue into thin ECM sheets was carried out through blending, pulverization, centrifugation, casting, freeze drying and SDS treatment. These sheets exhibited high porosity, pore connectivity and required mechanical properties of that of engineered scaffolds. Culturing of five different human cell types showed positive proliferation and integration into these decellularized ECM sheets.	169
29	human lipoaspirate	SDS, sodium deoxycholat e lipase and 500 U of porcine colipase treatment		0.1 M HCl and 3200 IU porcine pepsin used for digestion	The adipose matrix stripped off its cells shows a closer similarity in terms of biochemical composition and complexity to that of the native adipose ECM. The immunorejection concerns are minimized by the removal of both lipids and cellular contents, even if the allogenic source leads to formation of lipoaspirate. Small needle delivery is realized by its gelation property which takes place at body temperature itself, which further facilitates fine contouring of complex voids. Hence decellularized lipoaspirate produces an autologous soft tissue filler which is capable of gelling at body temperature and produces minimum immune response.	170
30	Human Adipose tissue ECM	Homogeniz ed Centrifugati on Repeated several times			Comparative study between a 3-D and a 2-D culture system highlighted the increase in the number of hASCs to upto 10 fold in the former against 2-8 fold expansion in the latter experiments. These hASCs retained their capability to differentiate into adipogenic, osteogenic and chondrogenic lineages as they positively expressed surface markers of pluripotency viz. Oct-4, Sox-2	171

					during 3-D culture.	
31	Human Adipose tissue ECM	(4–5 times) freeze-thaw digested in 0.5 M acetic acid	Solvent: 1,1,1,3,3,3-hexaflouro-2-propanol or 2,2,2-triflouro acetic acid 650RPM 2.5 mL/hr. +25 kV	Digested tissue dialyzed Lyophilized Scaffold cross-linking for 72 hr at RT in 30 mM genipin	The attachment and growth of ASC on electrospun At-ECM with similar results under blended scaffold with polydioxanone, suggesting that cultivation of ASC is higher in electrospun At-ECM scaffolds. The results of these studies validate the credibility of At-ECM scaffolds which can be isolated and electrospun as a basement membrane-rich matrix which can support stem cells. These results pave way for further future regenerative medicine studies.	172
32	Human Adipose tissue ECM	Mechanical disruption, polar solvent extraction inisopropanol, and enzymatic digestion (trypsin/EDTA, DNase, RNase, and lipase).		Decontaminated, digested with mixture of acetic acid/pepsin	Microcarrier fabrication methods were developed from decellularized adipose tissue (DAT) through non cytotoxic protocols. The efficacy of the fabrication protocols to produce stable beads as well as that of microporous surface topography was confirmed through microscopic characterization.	173
33	PLGA Type I collagen Porcine ECM from the SIS and UBS composite of PLGA and UBS	Washing, delaminatio n, peracetic acid/ ethanol treatment,		lyophilized UBS-ECM powder was uniformly mixed with PLGA to create produce composite.	Sites implanted with porcine ECM as compared to sites implanted to either Type I collagen or PLGA showed larger number of cells expressing Gpi-1. Recipient mice transplanted with marrow cells expressing β-galactosidase validated that majority of the population of cells which remodelled the naturally occurring porcine ECM were marrow derived. A significant increase in the number of marrow-derived cells which integrated into remodelled implant site were as a result of addition of porcine ECM to the PLGA scaffold.	174

34	Decellularized porcine dermis	Series of agitations involving organic solvents, detergents, salt solutions, enzymes and multiple rinsings.		Lyophilization, ethylene oxide sterilization. g.	In the post-processing examination it was found that various ECM proteins such as collagen III, collagen IV, collagen VII, laminin and fibronectin remained in the material. Glycosaminoglycans such as hyaluronic acid were also found to be present. Examination and quantification for the presence of cytokines showed more vascular endothelial growth factor (VEGF) and transforming growth factor b (TGF-b) as compared to decellularized dermis materials. Bioactivity retention was also demonstrated via cell culture assays.	175
35	Porcine UBM.	Delamination, peracetic acid/ ethanol treatment,		Powderisation: 1.The snap frozen material , reduced to small pieces 2. Salt (NaCl) mediated snap freezing and chopping Waring blender	Ground particles without salt precipitation when observed under SEM were found to be irregularly shaped sheet-like structured. The ones produced by salt precipitation method were found to be round and porous in appearance with a particle size range of 1mm which agglomerates with each other and bigger particles. It was concluded that the production of ECM particles of a uniform particle size depends on the manufacturing methodology	176
36	Porcine SIS, UBM, Liver	SIS & UBM: delamination, peracetic acid/ ethanol treatment, Liver stroma: trypsin, EDTA, Triton X-100, sodium deoxycholic acid, peracetic acid treatment.		--	The remaining DNA was shown to be present in smaller fragments according to the study, indicative of low possibility of these fragments playing a causative role in any of the adverse tissue remodelling response	177

3. MATERIALS AND METHODS

3.1. Procurement of Adipose Tissue

Human subcutaneous adipose tissues were harvested from patients undertaking during reduction mammoplasty or abdominoplasty procedures at the Seva clinic, Kolkata, India. Upon collecting the tissue, were stored in sterile plastic bags and stored at 4ºC and transported to to lab.

3.2. Decellularization of Adipose Tissue

The Cauterized portions parts of the harvested adipose tissue were discarded and the tissue washed in distilled water for 3-4 times to remove the blood components. The washed adipose tisuue mixed with double volume of distilled water and homogenized in house hold blender for 5min with pulsation in every 30 seconds. The oil expelled out from the cells during homogenization was separated by centrifuging the suspension at 3000*g* for 5 min at room temperature. The upper oil layer and lower water rich layer discarded and the middle layer containing adipose tissue and ECM stored for further processing. The sample after mechanical decellularization (homogenization), treated with freeze thawing method. The sample subjected to 15 cycles of freeze thawing (-20^{0}C- 37^{0}C), oil expelled after each cycles was removed by centrifugation at 3,000*g* for 5 min. After the freeze-thawing the samples were treated with anionic detergent sodium dodecyl sulfate (SDS) to remove the oil content and also help to break the plasma membrane and nuclear membrane thus will help to remove the immunogenic component DNA. The ECM stirred in 0.5% SDS solution for 1h at room temperature. The SDS trapped inside the ECM was removed by continuous washing in distilled water for 24h at 4^{0}C with a water replacement in 2hr gap. The SDS treated ECM were fabricated in to three dimensional scaffold by freeze drying method.

3.3. Evaluation of decellularization and delipidization

The extent of decellularization can be visualized by hematoxylin and eosin (H&E) staining. The tissue and decllularized tissue were fixed in paraformaldehyde and embedded in paraffin wax and sectioned in to 20 µm slices and the slides used for histological analysis. The procedure for H&E staining as follows.

- Flushed with hematoxylin for 10min
- Washed in running tap water for 5min
- Treated with acid-alcohol (1ml HCl + 49ml 70% ethanol) solution for 30s
- Washed in running tap water for 1min
- Blushed in ammonia water(0.2%) for 1min
- Washed in running tap water for 5 min
- Rinsed in 95% ethanol (10 dips)
- Counter stained the tissue with Eosin (1-5min)
- Washed in running tap water
- 100% ethanol treatment for 1 min
- Xylene: 100% ethanol treatment for 1 min
- Xylene treatment for 1 min
- Observed under simple microscope

The delipidization efficiency compared after each step of decellularization with Scanning Electron Microscopy SEM (JEOL JSM 6480LV SEM) analysis. The oil content in the ECM affects surface characteristics of lyophilized ECM that analyzed from SEM images.

3.4. Freeze drying of decellularized tissue

The decllularized tissue frozen at -80^{0}C for 24hr and were lyophilized in a freeze-dryer (Operon Chemical free freeze dryer-120C) under -115^{0}C and 650-950mmtorr for at least 48 hr.Scaffolds were then cross linked by UV (λ=254nm) irradiation for 15min and stored in air tight container.

3.5. Characterization of porous ECM scaffold

3.5.1. Scanning electron microscopy:

The morphology of obtained scaffolds was observed at both cross-section and longitude-section using SEM (JEOL JSM 6480LV SEM) after being coated with platinum powder at an acceleratingvoltage of 15 kV.

3.5.2. Fourier Transform Infra-Red (FTIR) spectroscopy:

The chemical functional groups were analyzed by Fourier transform infrared spectroscopy (FTIR, BRUKER) in the range of 500 – 3500 cm^{-1}. ECM Samples were milled with potassium bromide KBr and pressed into pellets before the FTIR analysis.

3.5.3.Visualization of elastic fibers by staining (Verhoeff-Von Gieson staining):

The sections of ECM after decellularization were stained to detect the presence of elastic fibers. The sections were first stained with Verhoeff's hematoxylin for 30 min. Samples were washed with distilled water and differentiated in 2% ferric chloride ($FeCl_2$) solution and observed under microscope for black fibers on gray background. Samples were washed again with water and treated with 5% hypo for 2 min to remove the iodine after decolorization washed again and counter stained with Van Gieson's for 5min. stained sections were dehydrated and examined microscopically for black elastic fibers and

red collagen fibers.

3.5.4. Swelling Ratio study

Swelling ratio indicates the affinity of scaffold to hold the water in its matrix that is proportional to hydrophilicity of scaffold. ECM scaffolds after lyophilization were weighed (W_d) and immersed in phosphate buffered saline PBS (, pH 7.4) at room temperature. The scaffolds were removed from PBS and blotted on filter paper to remove loosely bound water and wet weights of the scaffolds (W_t) were noted after a particular period of time (5, 10, 20, 30, 60, 120 min). The experiment was performed in triplicates. The swelling ratio (%) can be defined as the ratio of the weight increase ($W_t - W_d$) to the initial dry weight (W_d).

$$Swelling\ ratio\ (\%) = \frac{Wt - Wd}{Wd}\ \%$$

3.5.5. Biodegradability

Acellular ECM scaffolds were weighed (30±5mg) and then immersed in 10 ml autoclaved PBS containing 0.7U/ml collagenase IV (MP Biomedicals 195110) and incubated at 37°C with a gentle shaking. At fixed time intervals, scaffolds were removed from PBS, rinsed with water, lyophilized and weighed to measure the weight loss. The biodegradability can be calculated by using the equation:

$$Biodegradability\ (\%) = \frac{Wi - Wf}{Wi}\ \%$$

Where W_i is the initial weight and W_f is the weight after incubation time t.

3.6. Development of nHA by wet chemical method

The nHA was prepared by wet chemical method using Calcium nitrate $Ca(NO_3)_2 \cdot 4H_2O$ and Ammonium phosphate dibasic $(NH_4)_2HPO_4$ as Ca and P precursors, respectively.

$10Ca(NO_3)_2 + 6(NH_4)2HPO_4 + 8NH_4OH \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 20NH_4NO_3 + 6H_2O$

0.03 M aqueous solution of Ammonium phosphate dibasic was added drop wise to 0.05 M aqueous solution of calcium nitrate with vigorous stirring at 80±5 °C. The pH of calcium nitrate solution used for the reaction was adjusted to 11-12 with ammonia (NH_4OH). The nano HA (nHA) particles aged for overnight at room temperature. The sediment nHA particles were collected and washed with distilled water and harvested by centrifuging at 8000*g* for 10min. The powder dehydrated by freeze drying and calcinated to make it more crystalline at 600 °C for 1 h. (Fig 2)

3.6. Characterization of nHAP

3.6.1. X ray diffraction

The qualitative phase content of produced nHA were analyzed by by X-ray diffraction (XRD) by using a XRD diffractometer (X'Pert MPD Philips analyticl PANalytical 1.57A) with filtered 0.154056 nm Cu Kα radiation, in the scanning range $2\theta = 0 - 70^{o}$.The results obtained from the machine analyzed with X'Pert HighScore software package and identified the presence of characteristic peaks of nHA and crystal size also estimated.

3.6.2. FTIR Spectroscopy

Different functional groups present in the prepared nHA powder were studied with Fourier transform infrared spectroscopy FTIR by using FTIR-BRUKER Spectrometer. Prior to scanning nHA mixed with KBr powder (1:99) and pressed to 10mm diameter discs. The nHA disc were scanned in the range of 400-4000 cm-1.

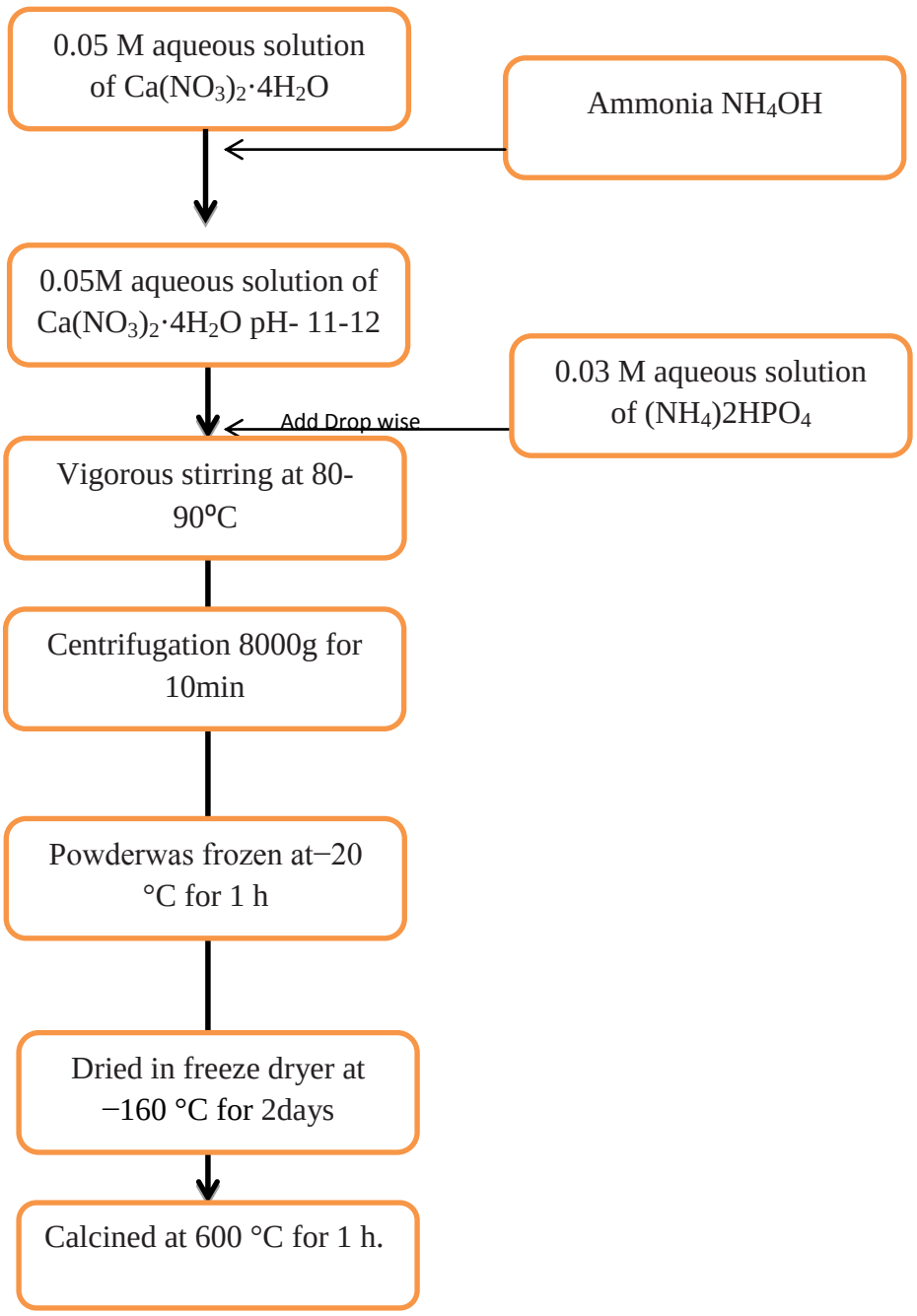


Fig 2: Schematic flow chart for the synthesis of nHA

3.6.3. Scanning electron microscopy (SEM) and EDS.

The approximate particle size can be measured from SEM images. nHA powder spread on carbon tape and observed in JEOL JSM 6480LV-type scanning electron microscope (SEM), operating at 25kV in vacuum. The energy dispersive X-ray attachment (EDAX/2001 device) attached to the SEM system can be used for elemental analysis of the powder.

3.6.4. Quasi-elastic light scattering (QELS) / Dynamic Light Scattering (DLS).

The size distribution profiles of nHA particles in suspension were studied using a Dynamic Light Scattering DLS (Zeta Sizer nano analyser Malvern). Before measuring particle size distribution, the suspensions (in water or 70% ethanol) were ultrasonicated the with a probe ultrasonicator**.**

3.7. ECM-nHAP Composite scaffold fabrication & Characterization

The ECM-nHA composites were synthesized using by adding the nHA particles to the digested ECM solution prior to lyophilisation (suspension method). ECM digested by stirring the matrix with 0.5M acetic acid (1:2) for 2 days. In the suspension method, nHA particles suspended in water were added into the digested ECM solution during the blending stage, followed by lyophilisation. Composite scaffolds containing 50 and 70% nHA prepared and characterized by XRD, FTIR, SEM and Mechanical Strength analyzer.

4. RESULTS AND DISUCSSION:

4.1. Decellularization

The adipose tissue harvested was red in color because of the presence of blood, and the presence of lipid-filled adipocytes makes the tissue oily and viscous. After decellularization the ECM become whitish and highly hydrated.

Fig 3: Red (Left): Adipose tissue before decellularization White (Right): Adipose tissue after decellularization

Blending procedure were found to be very effective decellularization method, because a large quantity of oil released after blending Fig: 4

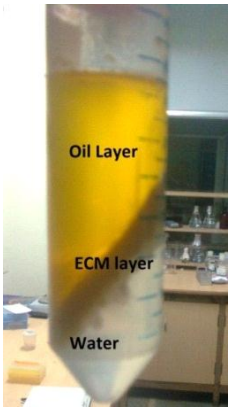


Fig 4: Oil separated after blending and subsequent centrifugation

4.2. Evaluation of decellularization and delipidization

H&E Staining:

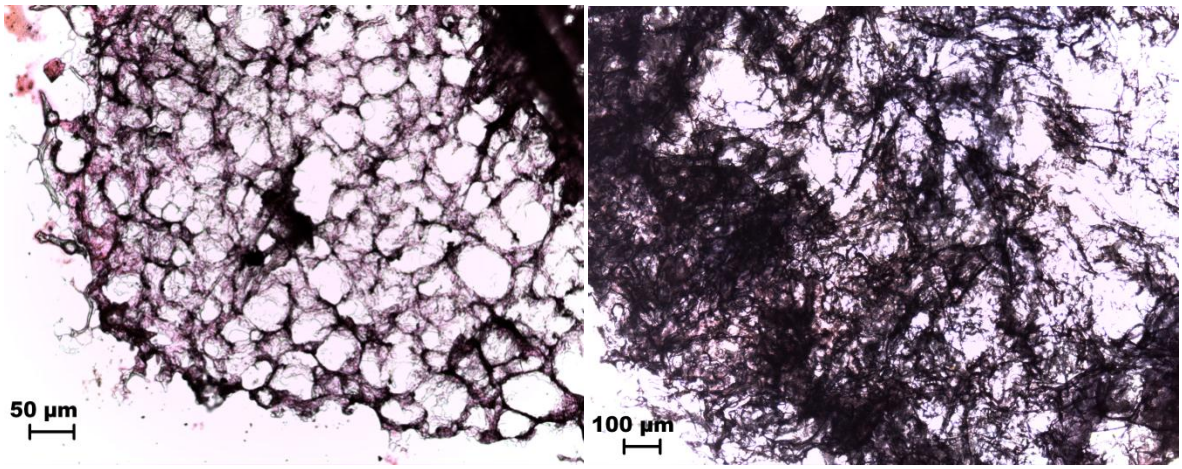


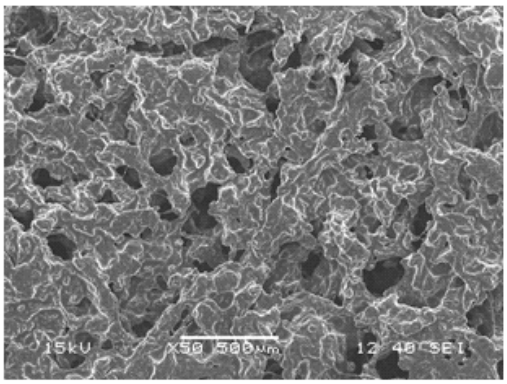
Fig 5: H&E Staining of Adipose tissue (right) and decllularized tissue (left)

H & E staining of tissue samples shown closed compartments these are adipose cells. But the H & E staining of decllularized matrix does not shown closed compartments, i.e. cells. H& E staining confirmed the absence of cells and cell debris in the decllularized (Fig.5)

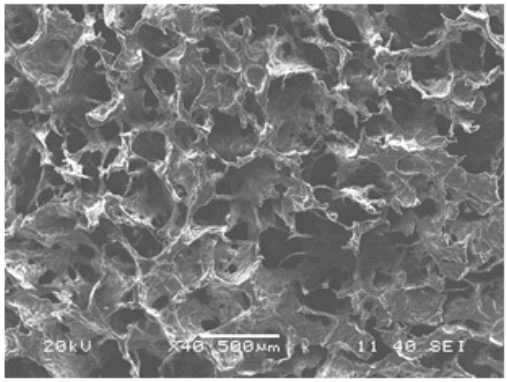
SEM analysis:

The SEM analysis of Scaffold obtained after different stages of decellularization are shown below (Fig:6).

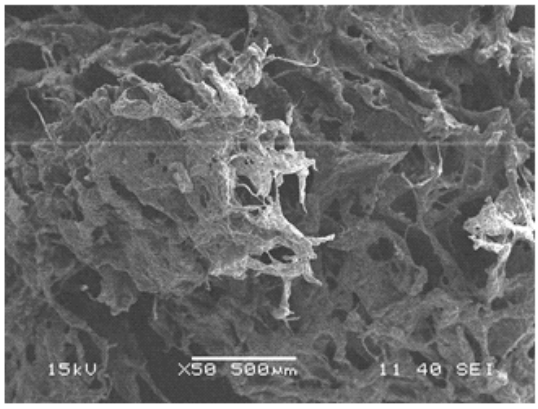
ECM surface after simple homogenization is an uneven globular appearance indicative of high lipid content. But the repeated freeze is thawing and SDS washing removed the lipid content.



SEM: After Homogenization



SEM: After Repeated freeze thawing (15 times) & Homogenization



SEM: After Repeated freeze thawing, Homogenization & SDS treatment

Fig.6. SEM results of ECM at different stages of decellularization process.

4.3. Freeze drying

After freeze drying a soft porous ECM scaffolds of different dimensions were obtained as shown in Fig: 7

Figure 7: Porous Scaffold after freeze drying

4.4. Characterization of ECM scaffold

4.4.1. Simple Microscopy

The scaffold shown highly porous structure, approximately 100-200µm pores were visible under the microscope (Fig.8)

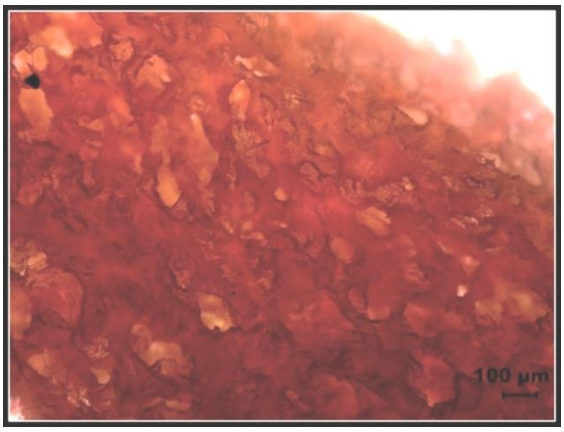


Fig 8: Light Microscopy of ECM Scaffold stained with eosin at 4X magnification

4.4.2. Scanning Electron Microscopy

Fig -9, 10 shows the SEM images of porous ECM scaffolds prepared from freeze drying method. The scaffold found to be highly porous and have interconnected pores also.

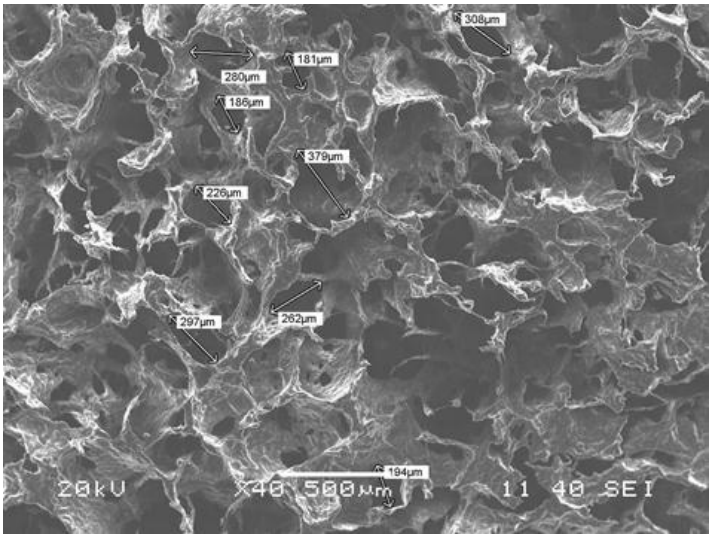


Figure 9: ECM Scaffold: Transverse cross section

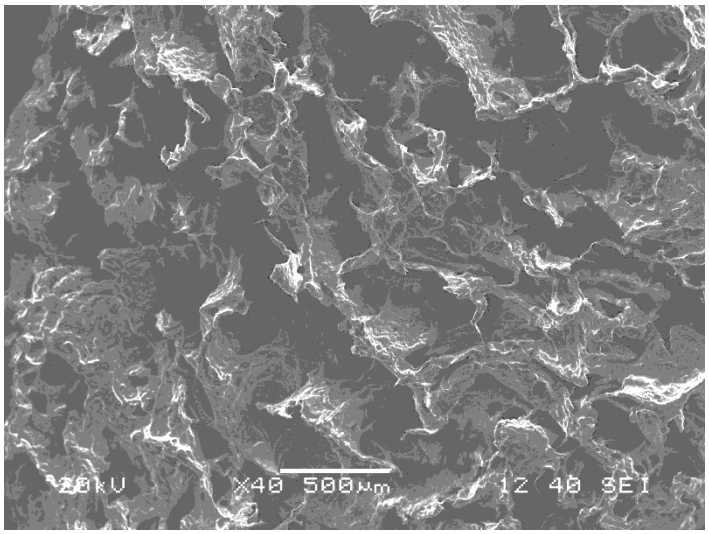


Figure 10: ECM Scaffold: Longitudinal section

From the SEM image it was observed that average pore size of ECM was 257 µm and pores were well interconnected.

4.4.3 Fourier Transform Infra-Red (FTIR) spectroscopy:

Characteristic wavenumber of following protein (Collagen) functional groups were found

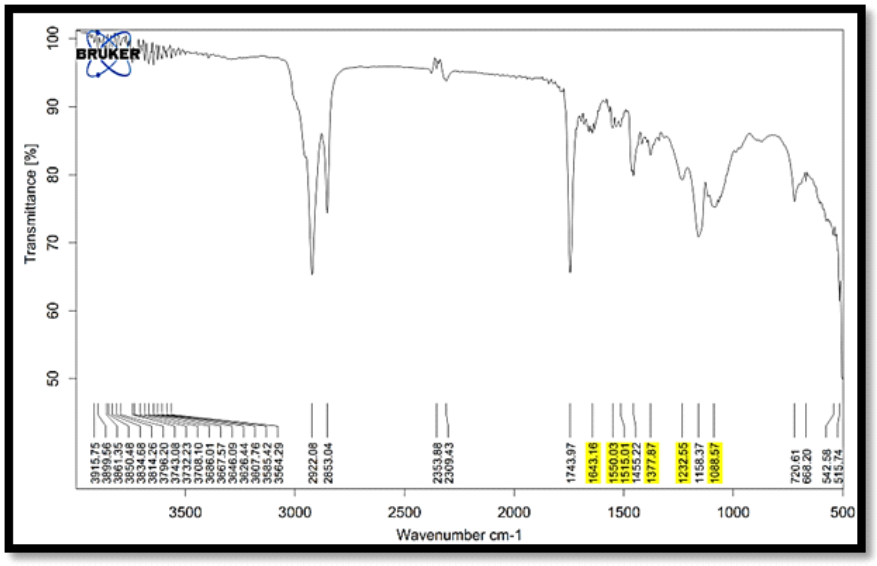


Figure 11: FTIR Result of ECM Scaffold

Table 5: ECM Functional groups and their wave number

Sl No:	Functional group	Wave number (cm^{-1})
1	Amide I region: mainly C=O , C-N bonds	1585-1720
2	Amide II region: mainly combination of C-N stretching and N- H bending vibrations	1500-1585
3	Amide III region: mainly C-N stretching, N-H bending, C-C stretching, and sulfate stretching vibrations	1200-1300
4	CH_2 bending vibrations, CH_3 asymmetric bending vibrations, COO^- stretching vibrations, and CH_2 side chains vibrations of collagen	1300 1500
5	CH_2 side chains vibration	1340
6	Carbohydrate region: stretching vibrations of C-O and C-OH, as well C-C ring vibrations	985-1140

4.4.4. Visualization of elastic fibers by staining (Verhoeff-Von Gieson staining):

The sample is stained with a regressive hematoxylin, consisting of ferric chloride and iodine. The differentiating is accomplished by using excess mordant (ferric chloride) to break the tissue-mordant dye complex. The dye will be attracted to the larger amount of mordant in the differentiating solution and will be removed from the tissue. The elastic tissue has the strongest affinity of the iron-hematoxylin complex and will retain the dye longer than the other tissue elements.

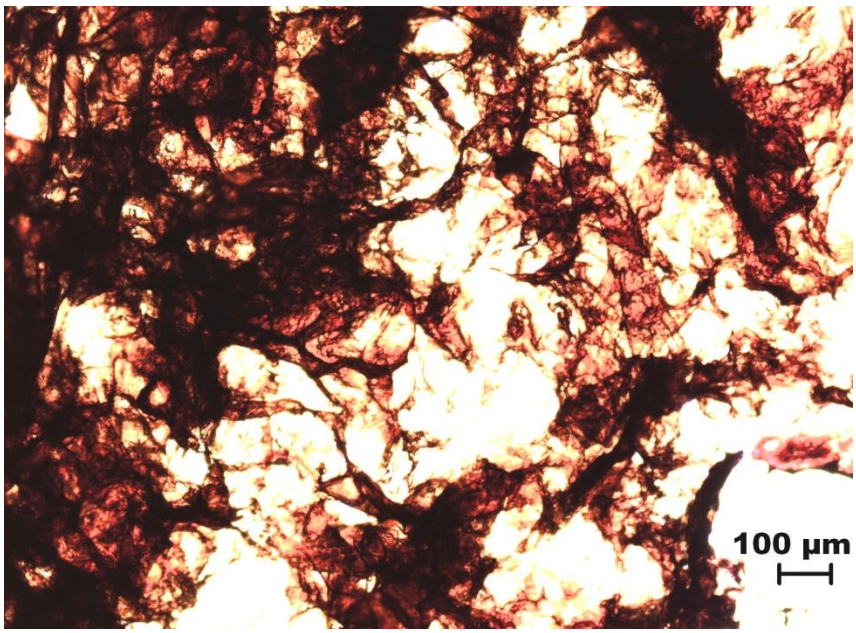


Fig: 12: elastic fibers by staining (Verhoeff-Von Gieson staining)

The ECM sections are rich in collagen (Red fibers) and elastic fibers (Black fibers) even after decellularization. It indicates that the decellularization method which we used did not affect the elastic protein much.

4.4.5. Swelling ratio

To determine the water retention or uptake property of the scaffolds which is one of the important properties required for scaffolds to be used in bone tissue engineering, the scaffolds were subjected for swelling studies as described in Section 2.4.3.

Table: 6: Swelling ratio data of ECM Scaffold

Sl No	Time (min)	Weight (g)	Swelling Ratio
1	0	0.0758	0
2	5	0.1149	0.5158
3	10	0.138	0.8206
4	20	0.2245	1.9617
5	30	0.321	3.2348
6	60	0.4008	4.2876
7	120	0.4153	4.4789
8	240	0.4164	4.4934

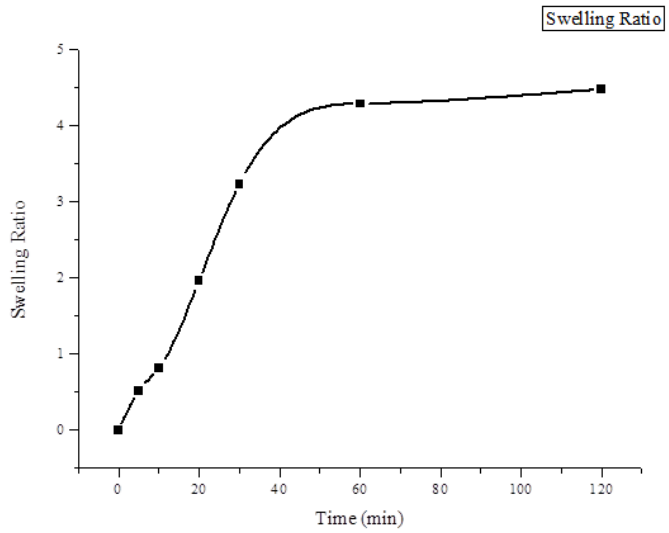


Figure 13: Swelling study of ECM scaffold

The swelling study indicated that the swelling ratio increased rapidly in first 1hr, after 1hr it was found to be almost constant value of approximately 430%. The increase in swelling ratio indicates that the increase in pore diameter and will help in easy cell infiltration.

4.4.6. Biodegradability:

Table 7: Biodegradability data of ECM Scaffold

Sl No	**No: days of incubation**	**Weight loss in %**	
		PBS	Collagenase + PBS
1	1	1.7	12.6
2	3	2.5	13.5
3	7	3.8	14.7
4	9	5	15.2
5	12	6.8	16.8
6	21	12.3	24.3
7	24	13.7	27.8

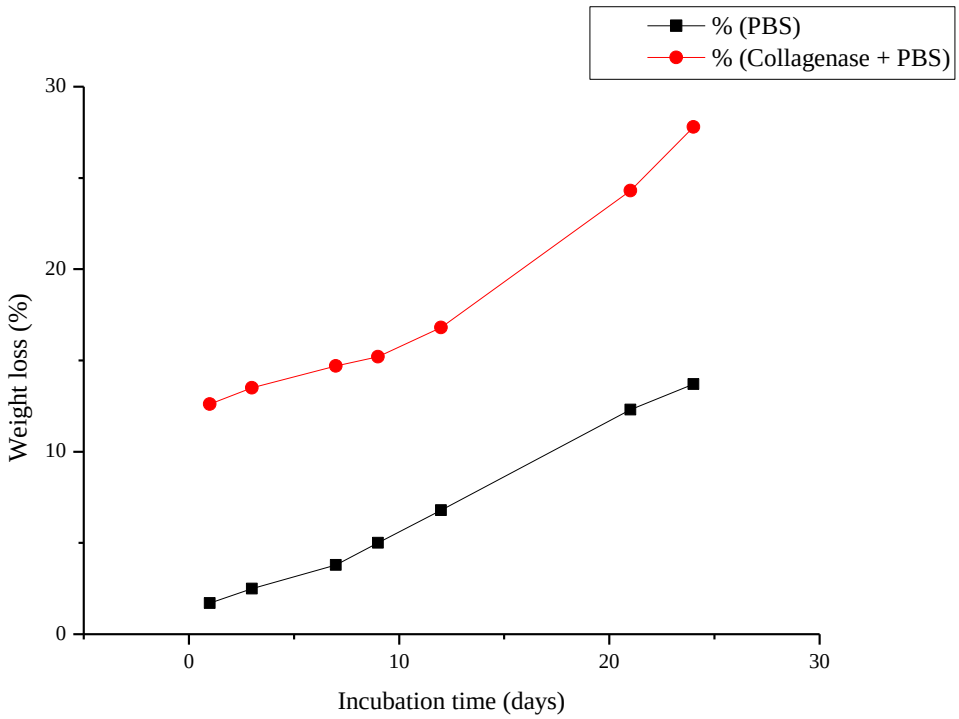


Figure 14: PBS and enzyme Biodegradation of ECM scaffold

From the graph it is clear that the ECM scaffolds are biodegradable in PBS also. The addition of enzyme collagenase increases the degradation rate; it indicates that the scaffold is rich in collagen molecules.

Fig. 15 shows SEM micrographs of various scaffolds after 21 days of incubation in PBS. From the images it's clear that the pores get degraded by enzyme collagenase is more effective than PBS. It indicates that the scaffold mainly contain collagen protein.

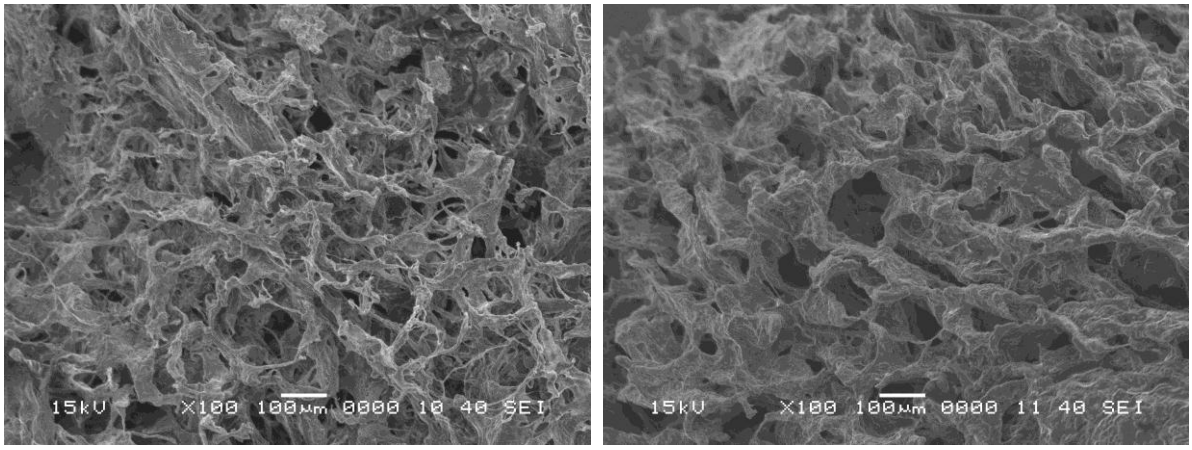


Fig: 15 SEM images of PBS (left) and Collagenase (Right) degraded scaffolds/

4.5: Nano Hydroxyapatite nHA- characterization

4.5.1: XRD Result

Figure 16 shows the XRD patterns of the nHA powders. Characteristic peaks of nHA (2Θ = 25.76°, 28.16°, 31.86°, 40.04°) were found in the spectrum by X'Pert Highscore software.All peaks were corresponding to hydroxy apatite and no additional peaks were found. The result indicates that the synthesized HA powder was phase pure.

Nanoparticle crystal size can be estimated by the following equation:

$$\text{Particle Size } D = \frac{0.9\,\lambda}{\beta\,cos\Theta} = \mathbf{10.48nm}$$

λ : Wavelength of X ray used ($1.54A^0$)

β: Wavelength of the full width at half maximum (FWHM=0.7872)

Θ: theta value correspond to maximum intensity peak (15.8421)

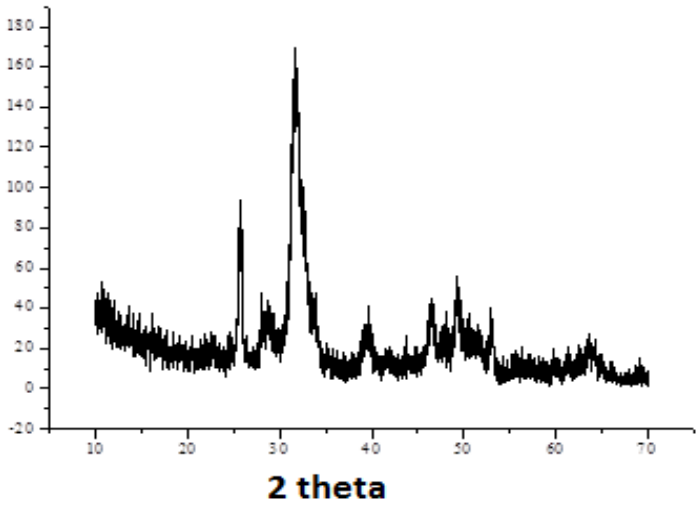


Figure 16 : XRD Result of nano hydroxy apatite

4.5.2. FTIR

Figure 17: shows FTIR spectra of the nHA. The wave number of bands and their assignments are summarized in Table.

Table 8: Characteristic wave number of powder HA

Sl No:	Functional group	Wave number (cm^{-1})
1	$\nu_2 (PO_4^{3-})$	470
2	$\nu_4 (PO_4^{3-})$	550, 600
3	$\nu_1(PO_4^{3-})$	960
4	$\nu_3(PO_4^{3-})$	1040, 1100
5	$\nu_L (OH^-)$	632
6	$\nu_3(OH^-)$	3572
7	$\nu_2(CO_3^{2-})$	860
8	$\nu_3(CO_3^{2-})$	1470

9	ν (NO_3^{2-})	1400
10	ν(OH^-) adsorbed water	1640, 3450

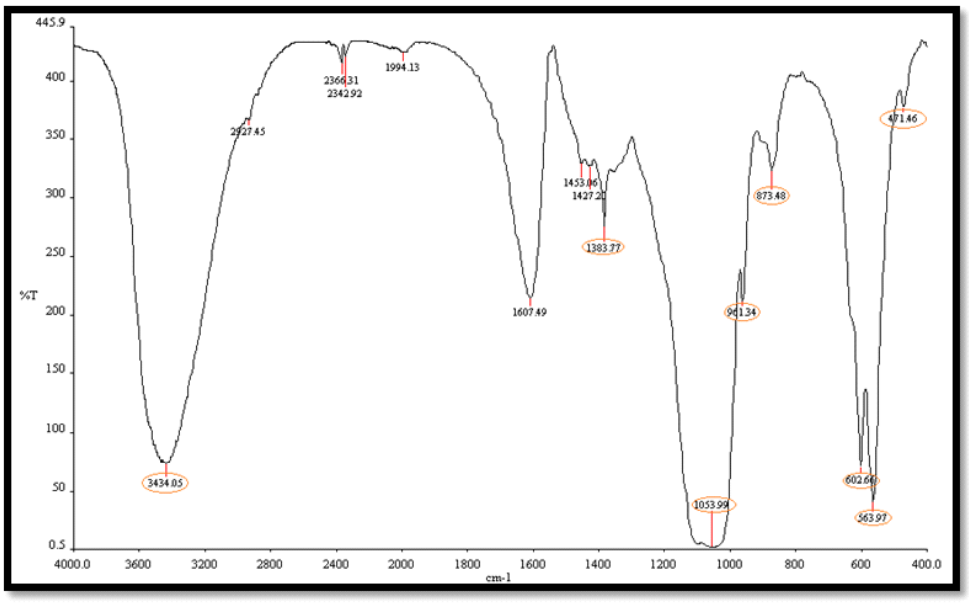


Fig: 17: FTIR Spectrum of nHA

4.5.3 Dynamic Light Scattering (DLS)

The size distribution profile of nHA particles in suspension is shown in Fig: 18, the single peak in the data indicates that all aggregate are of same range. The average size of the aggregates in the water suspension is 902nm.

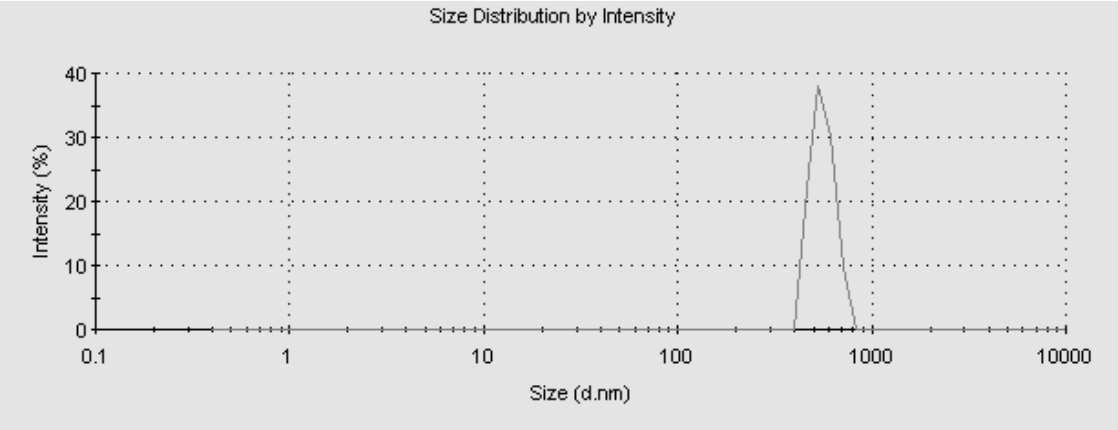


Fig: 18: Dynamic Light Scattering Result of nHA

4.5.4. SEM-EDS of nHA

The SEM analysis of nHA powder is shown in Fig: 19, from the SEM it is clear that the particles are very small (did not go for more magnification because of machine disability)

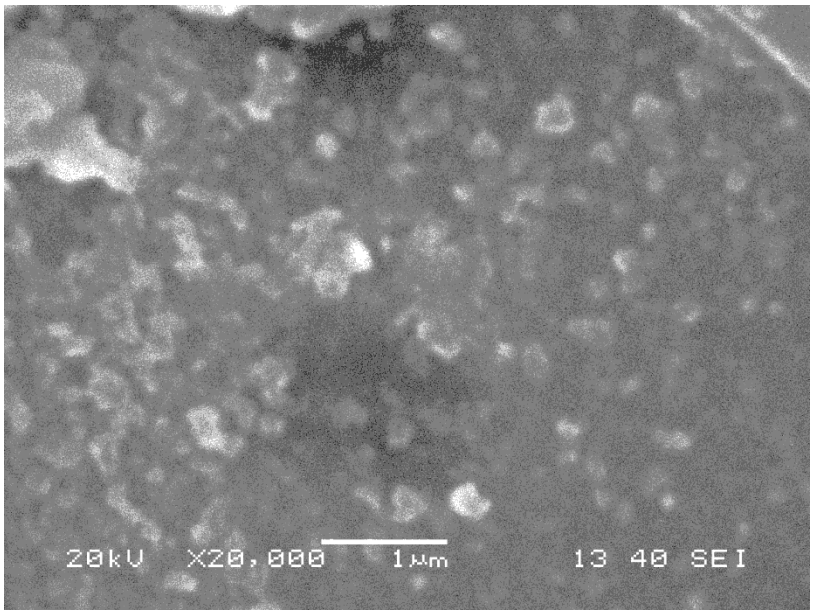


Fig: 19 SEM result of nHA

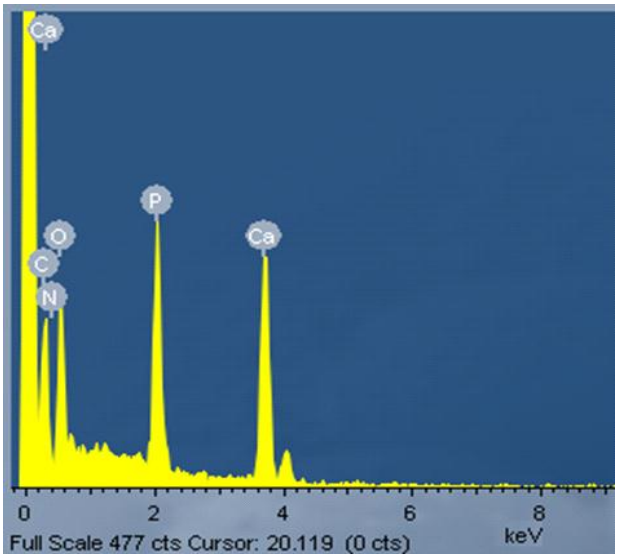


Fig: 20 : EDS of nHA

The EDS results (Fig: 20) shown that the compound produced possess all the elements of HA (Ca_{10} $(PO_4)_6(OH)_2$) like Ca, P, N,O. the presence of Carbon might be an impurity developed during the synthesis.

4.6: ECM-nHA composite scaffold characterization

ECM-nHA composite scaffolds were successfully synthesized by using the suspension method as shown in Fig: 21

Fig: 21: ECM-nHAP composite scaffolds (70%nHA Scaffold)

The scaffold containing 50% nHA shows more strength than simple ECM scaffold, but its very weak whrn it compare with scaffold having 70% nHA.

4.6.1. SEM:

ECM 50%nHA: Composite scaffold containing 50% nHA gave porous structure with average pore size 110 µm but from images its cleat that scaffold is not strong enough looks like puffy scaffold.(Fig:22)

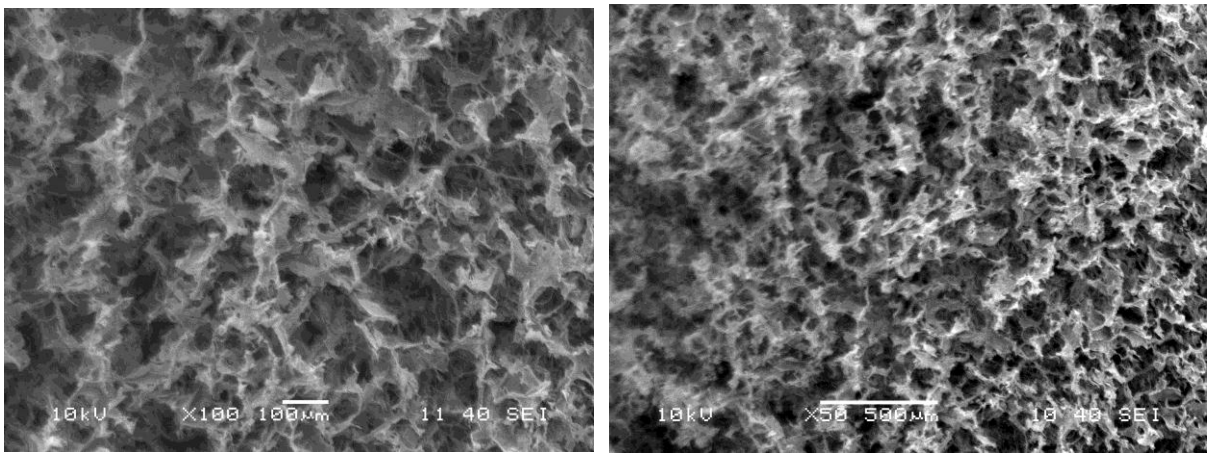


Fig: 22: SEM composite scaffold (50% nHA)

ECM 70%:ECM scaffold with 70% shown highly porous structure with an average pore size of 170 µm which is suitable for bone tissue engineering.

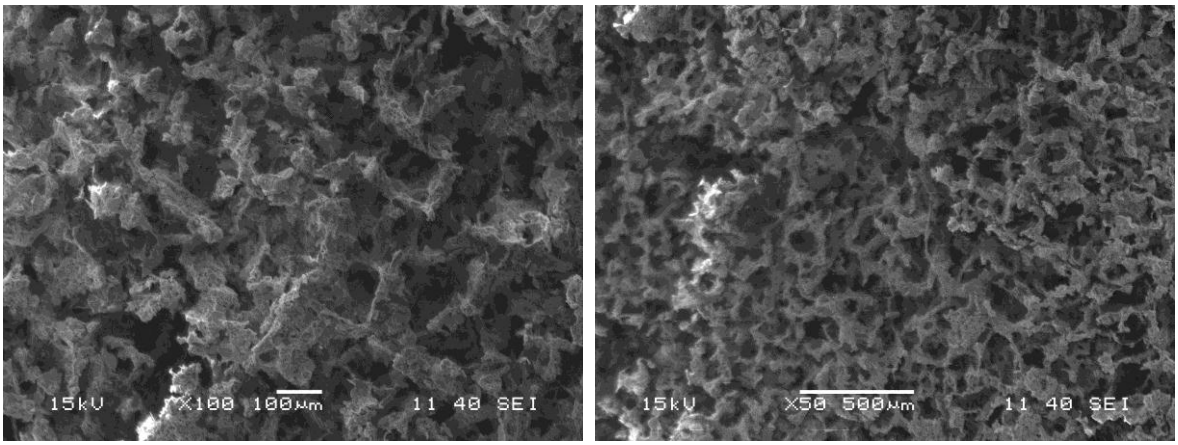


Fig: 23: SEM composite scaffold (70% nHA)

4.6.2. FTIR:

FTIR analysis of composite scaffold showed the characteristic peaks of both nHA and ECM.

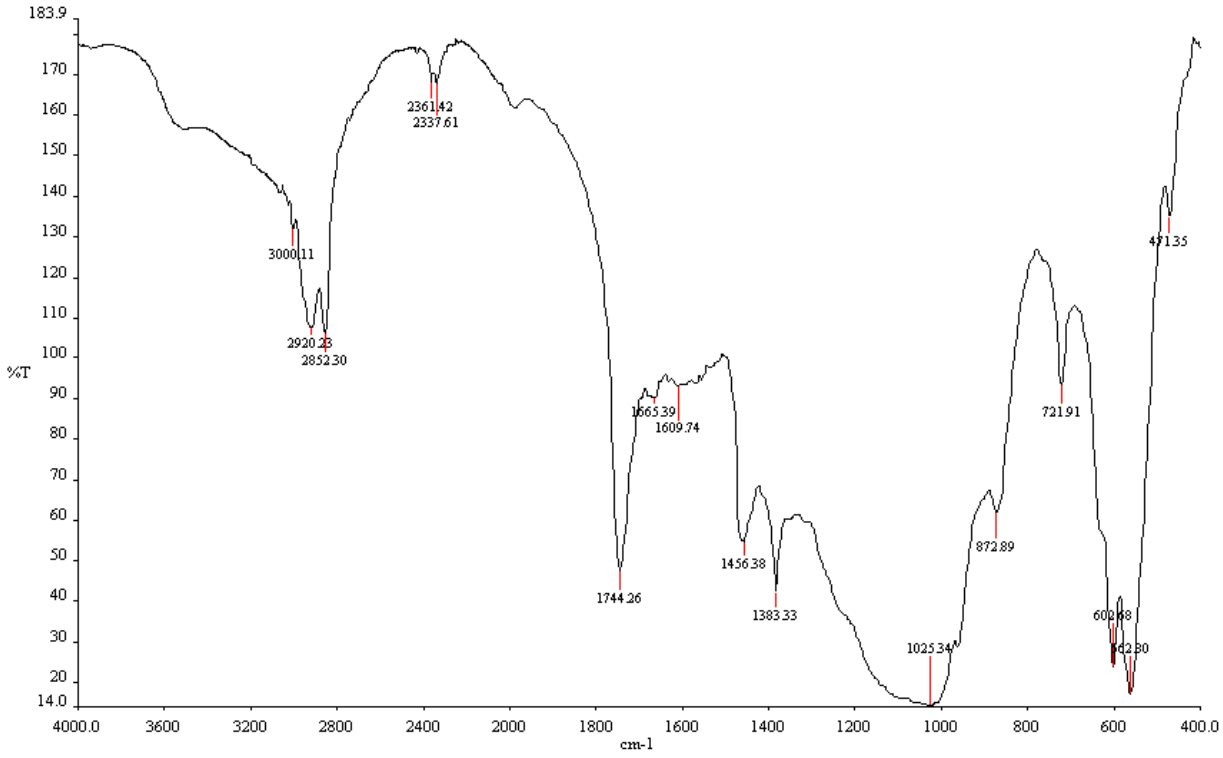


Fig: 24: FTIR composite scaffold (70% nHA)

4.5.1. XRD Results

The XRD results of composite shown all characteristics peaks of nHAP, it indicates that nHAP incorporated properly in the scaffold. (Fig: 25)

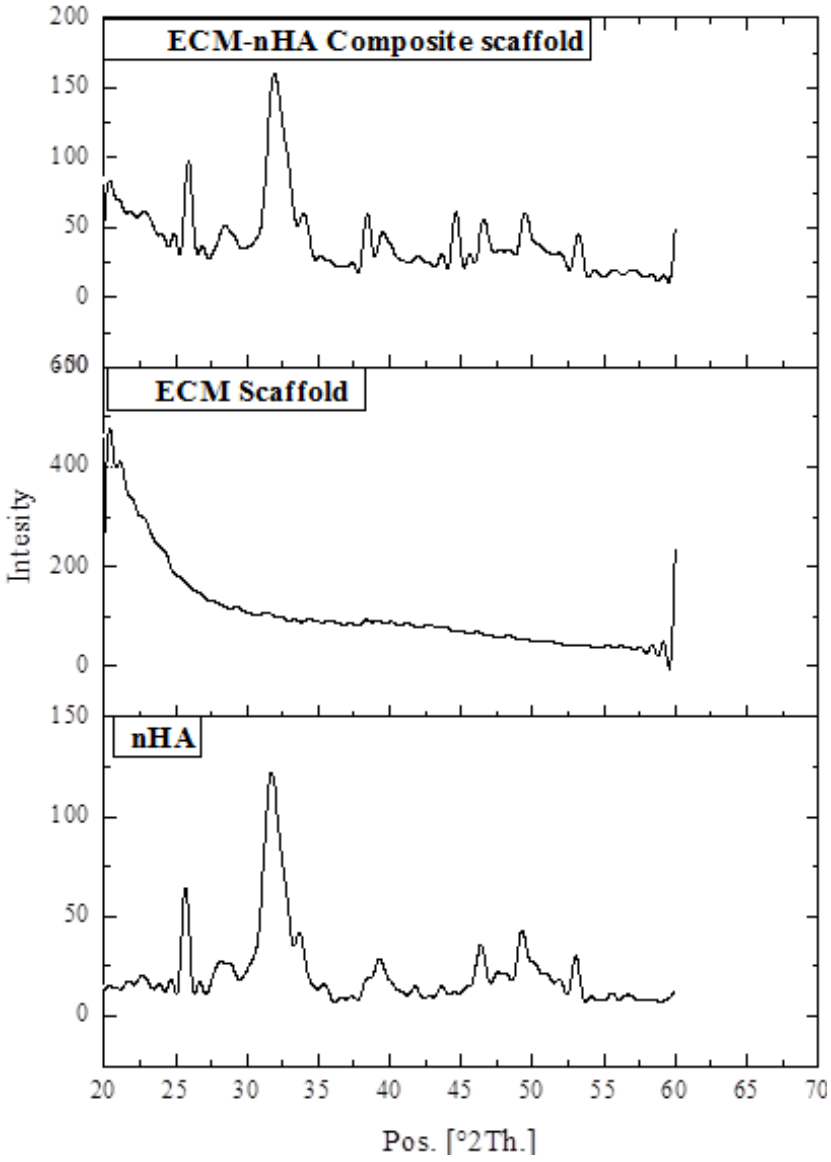


Fig: 25: XRD- ECM, nHA and composite scaffold (70% nHA)

5. CONCLUSIONS AND FUTURE PLANS:

In the current study optimized a Decellularization process by homogenization, freeze thawing & SDS treatment. The freeze drying of decellularized tissue gives a highly porous and hydrophilic scaffold which is suitable for soft tissue engineering. We can develop hydroxyapatite nano particles of 10nm size by simple wet chemical method which is suitable for bone tissue engineering. The composite scaffold developed by suspension method with acetic acid digested ECM showed good porous structure and mechanical strength which is desirable for bone regeneration applications.

In future we are planning to perform following ideas:

- Optimization of Composite scaffold fabrication for bone tissue applications
- In- vitro expansion of human adipose -derived stem cells in the developed scaffolds
- In- vivo study of the fabricated scaffold in animal model.
- Clinical trial experiments
- Marketing the product

REFERENCE:

1. Times of india.com, http://articles.timesofindia.indiatimes.com/2010-06-07/india/28314801_1_hip-fractures-asian-audit-bad-bone-health (20 May 2013)
2. M.J.Yaszemski,J.B.Oldham,L.Lu,B.L.Currier, Bone Engineering , *Em squared, Toronto*., (1994), pp541.
3. R.Spitzer, C.Perka, K.Lindenhayn, H.Zippel, *J.Biomed.Mater.Res*., 59 (2002), pp-,690.
4. G. C. Simon, C. A. Khatri, S. A. Wight, F. W. Wang, *J.Orthop.Res*., 20 (2002), pp-473.
5. F.R.Rose,R.O.Oreffo, *Biochem.Biophys.Res.Commun*.(2002), pp-292
6. H.Petite,V.Viateau,W.Bensaid,A.Meunier,C.dePollak,M. Bourguignon, K. Oudina, L. Sedel, G. Guillemin,*Nature Biotech*., 18, (2000),pp-959.
7. I.Asahina,I.Seto,M.Oda,E.Marukawa,A.M.Imranul, S. Enomoto, Bone Engineering, *Em squaredToronto*.,1, (1999), pp-526.
8. G. P. Reece, C. W. Patrick, Jr., Frontiers in Tissue Engineering, *Pergamon, Oxford*., 1,(1998),pp-166.
9. D. F. Williams, Bone Engineering, *Em squared, Toronto.*,1,(1999), 577.
10. Lutolf MP, Hubbell JA, Synthetic biomaterials as instructive extracellular micro environments for morphogenesis in tissue engineering. *Nat Biotechnol*., 23, (2005), pp- 47–55.
11. Mariman ECM, Wang P, Adipocyte extracellular matrix composition, dynamics and role in obesity, *Cellular and Molecular Life Sciences.*,67(2010), pp-1277-92
12. Kadler, K.E, Extracellular matrix 1: fibril-forming collagens. *In: Protein Profile*.,2 (1995),pp- 491-619

13. Kuhn, K. ,The classical collagens: types I, II and LU. In: Structure and Function of Collagen 'Ifipes, ed. Mayne, R. and Burgeson, R.E., Academic Press, London, (1987), pp. 1-42.
14. J. Rossert, B. de Crombrugghe, Type I collagen: structure, synthesis and regulation, in: J.P. Bilezkian, L.G. Raisz, G.A. Rodan (Eds.), Principles in Bone Biology, Academic Press, Orlando, 2002, pp. 189–210.
15. Liu, X. III collagen is crucial for collagen I fibrillogenesis and for normal cardiovascular development. Proc. Natl Acad. Sci. USA 94, 1997: pp- 1852- 1856.
16. K. Gelse, E. Po¨schl, T. Aigner., Collagens—structure, function, and biosynthesis, *Advanced Drug Delivery Reviews*., 55, (2003), pp-1531– 1546
17. Soininen, R. Complete primary structure of the al-chain of human basement membrane (type IV) collagen. FEBS Lett. 225: 1987, pp-188-194.
18. Hostikka, S.L. and Tryggvason, K, The complete structure of the a2 chain of human type IV collagen and comparison with the al(IV) chain. *J. Biol. Chem*. 263 (1998), pp-19488-19493.
19. Fichard, A, another look at collagen V and XI molecules. *Matrix Biol*. 14, (1995): pp-515-531
20. Keene, D. R., L. Y Sakai, and R. W. Glanviile.. Ultrastructure of type VI collagen in human skin and cartilage suggests an anchoring function for this filamentous network. *J. Cell Biol*. 107(1988):pp-1995-2006.
21. Bonaldo, P., V. Russo, F. Buccioti, R. Doliana, and A. Colombatti.. Structural and functional features of the c~3 chain indicate a bridging role for chicken collagen VI in connective tissues. *Biochemistry*. 29(1990), pp-1245-1254.
22. Evered, D., and J. Whelan. The Biology of Hyaluronan. Ciba Foundation Symposium 143.1. Wiley, Chichester, UK.(1988.)

23. Burgeson, R.E. Type W collagen. In: Structure and Function of Collagen Types, ed. Mayne, R. and Burgeson, R.E., Academic Press, London, (1987) ,pp. 145-172.
24. Shuttleworth, C.A. Type VIII collagen. *Int. J. Biochem. Cell Biol*. 28: (1997) , pp-1-4.
25. Sutmuller M, Bruijn JA, de Heer E. Collagen types VIII and X, two non-fibrillar, short-chain collagens. Structure homologies, functions and involvement in pathology.,*Histol Histopathol*. 12 1997; pp-557-66.
26. Reichenberger, E. and Olsen, B.R. Collagens as organizers of the extracellular matrix during morphogenesis. Semin. *Cell Develop. Biol*. 7: (1996) ,pp-631-638.
27. Werner Miiller-Glauser, Bruno Humbel, Markus Glatt, Peter Str/iuli,Kaspar H. Winterhalter, and Peter Bruckner, On the Role of Type IX Collagen in the Extracellular Matrix of Cartilage: Type IX Collagen Is Localized to Intersectionsof Collagen Fibrils, *The Journal of Cell Biology*, 102, (1986), pp-1931-1939
28. Sutmuller M, Bruijn JA, de Heer E. Collagen types VIII and X, two non-fibrillar, short-chain collagens. Structure homologies, functions and involvement in pathology.,*Histol Histopathol*. 12, (1997), pp-557-66.
29. Fichard, A. et al. Another look at collagen V and XI molecules. *Matrix Biol*. 14: (1995) ,pp- 515-531.
30. Walchli, C. Complete primary structure of chicken collagen XIV. *Eur. J. Biochem.* 212: (1993), pp-483-490.
31. Lai, C.-H. and Chu, M.-L. Tissue distribution and developmental expression of type XVI collagen in the mouse. *Tissue Cell* 28: (1996),pp-155-164.
32. C.H. Lai, M.L. Chu, Tissue distribution and developmental expression of type XVI collagen in the mouse, *Tissue Cell* 28

33. Rehn, M. Primary structure of the a1 chain of mouse type XwI collagen, partial structure of the corresponding gene, and comparison of the al(XVIII) chain with its homologue, the al(XV) collagen chain. *J. Biol. Chem*. 269: (1994) ,pp-13929-13935.
34. Myers, J.C. The triple-helical region of human type XIX collagen consists of multiple subdomains and exhibits limited sequence homology to al(XV1). *J. Biol. Chem*. 269: (1994) ,pp-18549-18557.
35. Sandberg, L.B. and Davidson, J.M. Elastin and its gene. *Peptide Protein* Rev. 3: (1984) pp- 169-193.
36. Timpl, R. and Brown, J.C. The laminins. *Matrix Biol*. 14: (1994) ,pp- 275-281.
37. akai, L.Y. Fibrillin, a new 350-kD glycoprotein, is a component of extracellular rnicrofibrils. *J. Cell Biol*. 103: (1986) , pp- 2499-2509.
38. Mariencheck, M.C. Fibrillin-1 and fibrillin-2 show temporal and tissue-specific regulation of expression in developing elastic tissues. *Conn. Tiss. Res*. 31: (1995) , pp-87-97.
39. Heinegird, D. Two novel matrix proteins isolated from articular cartilage show wide distributions among connective tissues. *J. Biol. Chem.* (1986) pp-124-127
40. Dudhia, J. and Hardmgham, T.E. Primary structure of human cartilage- link protein. *Nucleic Acids Res.* 18: (1990) , pp- 1292.
41. Luo, G. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature* 386: (1997) ,pp-78-81.
42. Murphy, G. and Knauper, V. Relating matrix metalloproteinase structure to function: why the 'hemopexin' domain? *Matrix Biol*. 15: (1997) ,pp- 511-518.
43. Paulsson, M. Laminin-nidogen complex. Extraction with chelating agents and structural characterisation. *Eur. J. Biochem*. 166: (1987) , pp-467-478.

44. Kallunki, P. and Tryggvason, K. Human basement membrane heparan sulphate proteoglycan core protein: A 467-kD protein containing multiple domains resembling elements of the low density lipoprotein receptor, laminin, neural cell adhesion molecules and epidermal growth factor. 1. *Cell Biol*. 116: (1992), pp- 559-571.
45. aldwrn, C.T new eptderrtlal growth lactor-like repeat m the human core protein !or the Iage cartilage-cpcctfic pruteogiycan *j. Biol Chem*, 264, (1989), pp- 15747-15753.
46. Couchman, J.R. Perlecan and BM-CSPG (Bamacan) are two basement membrane chondroitin/dermatan sulphate proteoglycans in the Engelbreth-Holm-Swarm tumor matrix. *J. Biol. Chem*. 271: (1996), pp- 9595-9602.
47. Roughley, P.J. and White, R.J. Dermatan sulphate proteoglycans of human articular cartilage. The properties of dermatan sulphate proteoglycans I and II. *Biochem. J.* 262: (1989), pp-823-827.
48. Chen, Q. Progression and recapitulation of the chondrocyte differentiation program: Cartilage matrix protein is a marker for cartilage maturation. *Develop. Biol*. 172: (1995), pp-293-306.
49. Neamc, P.J. The structure of a 3X-kDa leucinc-rich protein (chondroadherin] isolated from bovine cartilage. *J. Biol. Chem*. 269, (1994) , pp-21547-21554.
50. Robey, P.G., Vertebrate mineralized matrix proteins: structure and function. *Connect. Tiss. Res*. 34: (1996) ,pp-269-276.
51. Butler, W.T , Extracellular matrix proteins of dentine. *CIBA Foundation Symp*. 205: (1997) , pp-107- 1 17.
52. Pierschbacher, M.D. and Ruoslahti, E. The cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* 309: (1984) , pp-30-33.

53. Argraves, W.S., Fibulin, a novel protein that interacts with the fibronectin receptor /3 subunit cytoplasmic domain. *Cell* 58: (1989), pp- 623-629.
54. Allison E.B. Turner, Claire Yu, JuaresBianco, John F. Watkins, Lauren E. Flynn. The performance of decellularized adipose tissue microcarriers as an inductivesubstrate for human adipose-derived stem cells. *Biomaterials* 33 (2012), pp- 4490-4499
55. BeobSoo Kim , Ji Suk Choi, Jae Dong Kim ,Young Chan Choi , Yong Woo Cho. Recellularization of decellularized humanadipose-tissue-derived extracellular matrix sheets with other human cell types. *Cell Tissue Res*, 348(2012), pp- 559-67
56. A.V. Piterina, L.M. Davis, C.L. Meaney, A.J. Cloonan, M.T. Walsh and Prof. Tim.M. McGloughlin.Cell-Seeded Decellularised Extracellular Matrices As An Advanced Approach For Tissue Engineering.Eccomas – *International Conference On Tissue Engineering*, 2009
57. Aidan J. Cloonan, Michael R. O'Donnell, William T. Lee, Michael T. Walsh, EamonnDeBarra, Tim M. McGloughlin. Spherical Indentation of free-standing acellular Extracellular Matrix Membranes, 2009.
58. Tottey S, Johnson SA, Crapo PM, Reing JE, Zhang L, Jiang H,. The effect of source animal age upon extracellular matrix scaffold properties. *Biomaterials* 32, (2011) : pp-128-36.
59. Raghavan D, Kropp BP, Lin H-K, Zhang Y, Cowan R, Madihally SV. Physical characteristics of small intestinal submucosa scaffolds are location-dependent. Journal of *Biomedical Materials Research Part A* , ;73A (2005), pp-90-96.
60. Badylak SF, Freytes DO, Gilbert TW. Extracellular matrix as a biological scaffold material: Structure and function. *Acta Biomaterialia* 5 (2009), pp-1-13.

61. Freytes DO, Stoner RM, Badylak SF. Uniaxial and biaxial properties of terminally sterilized porcine urinary bladder matrix scaffolds. *J Biomed Mater Res B Appl Biomater,* 84 (2008), pp-408-414
62. Badylak SF, Wu CC, Bible M, McPherson E. Host protection against deliberate bacterial contamination of an extracellular matrix bioscaffold versus Dacron mesh in a dog model of orthopedic soft tissue repair. *J Biomed Mater Res B Appl Biomater,* 67, (2003), pp-648–54.
63. Jernigan TW, Croce MA, Cagiannos C, Shell DH, Handorf CR, Fabian TC. Small intestinal submucosa for vascular reconstruction in the presence of gastrointestinal contamination. *Ann Surg,* 239, (2004), pp-733–8;
64. Shell DH 4th, Croce MA, Cagiannos C, Jernigan TW, Edwards N, Fabian TC. Comparison of small-intestinal submucosa and expanded polytetrafluoroethylene as a vascular conduit in the presence of gram-positive contamination. *Ann Surg,* 241, (2005), pp-995–1001.
65. Sarikaya A, Record R, Wu CC, Tullius B, Badylak S, Ladisch M. Antimicrobial activity associated with extracellular matrices. *Tissue Eng*, 8, (2002), pp-63–71.
66. Badylak, S.F., Wu, C.C., Bible, M., and McPherson, E. Host protection against deliberate bacterial contamination of an extracellular matrix bioscaffold versus Dacron mesh in a dog model of orthopedic soft tissue repair. *J Biomed Mater Res B Appl Biomater* , 67, (2003), pp-648
67. Badylak, S.F., Coffey, A.C., Lantz, G.C., Tacker, W.A., and Geddes, L.A. Comparison of the resistance to infection of intestinal submucosa arterial autografts versus polytetra fluoroethylene arterial prostheses in a dog model. *J Vasc Surg* 19, (1994) ,pp-465

68. Jernigan, T.W., Croce, M.A., Cagiannos, C., Shell, D.H., Handorf, C.R., and Fabian, T.C. Small intestinal submucosa for vascular reconstruction in the presence of gastrointestinal contamination. *Ann Surg* 239, (2004), pp-733,
69. Shell, D.H.t., Croce, M.A., Cagiannos, C., Jernigan, T.W., Edwards, N., and Fabian, T.C. Comparison of small-intestinal submucosa and expanded polytetrafluoroethylene as a vascular conduit in the presence of gram-positive contamination. *Ann Surg* 241, (2005), pp-995.
70. Mantovani, F., Trinchieri, A., Castelnuovo, C., Romano, A.L., and Pisani, E. Reconstructive urethroplasty using porcine acellular matrix. Eur Urol 44, 600, Badylak, S.F., Coffey, A.C., Lantz, G.C., Tacker, W.A., and Geddes, L.A. Comparison of the resistance to infection of intestinal submucosa arterial autografts versus polytetra fluoroethylene arterial prostheses in a dog model. *J Vasc Surg* 19,(1994), pp-465.
71. Zantop, T., Gilbert, T.W., Yoder, M.C., and Badylak, S.F. Extracellular matrix scaffolds are repopulated by bone marrow-derived cells in a mouse model of achilles tendon reconstruction. *J Orthop Res* 24,(2006),pp-1299..
72. Badylak, S.F., Park, K., Peppas, N., McCabe, G., and Yoder, M. Marrow-derived cells populate scaffolds composed of xenogeneic extracellular matrix. *Exp Hematol* 29, (2000), pp-1310,
73. Beattie, Allison J., Thomas W. Gilbert, Juan Pablo Guyot, Adolph J. Yates, and Stephen F.Badylak.Chemoattraction of progenitor cells by remodeling extracellular matrix scaffolds.(Report). *Tissue Engineering, Part A: Tissue Engineering*. 15 (2009), pp-1119

74. Guilak F, Cohen DM, Estes BT, Gimble JM, Liedtke W, Chen CS. Control of stem cell fate by physical interaction with the extracellular matrix. *Cell stem cell,* 5 (2009): pp-17-26
75. Cortiella J, Niles J, Cantu A, Brettler A, Pham A, Vargas G,. Influence of acellular natural lung matrix on murine embryonic stem cell differentiation and tissue formation. *Tissue eng Part A,* 16(2010), pp-2565-80
76. Ng SL, Narayanan K, Gao S, Wan AC. Lineage restricted progenitors for the repopulation of decellularized heart. *Biomaterials,* 32 (2011), pp-7571-80
77. Li, Y.Y., McTiernan, C.F., and Feldman, A.M. Interplay of matrix metalloproteinases, tissue inhibitors of metalloproteinases and their regulators in cardiac matrix remodeling. *Cardiovasc Res* 46,(2000), pp-214.
78. Lochter, A., and Bissell, M.J. Involvement of extracellular matrix constituents in breast cancer. *Semin Cancer Biol* 6,(1995), pp-165
79. oasiswoundmatrix.com, http://www.oasiswoundmatrix.com/bm, (6 Feb 2013)
80. teibio.com. http://www.teibio.com/products/by-brand/tissuemend/(6 Feb 2013)
81. mmi.ourassets.net, http://mmi.ourassets.net/?page=products_foot(6 Feb 2013)
82. synthes.com,http://www.synthes.com/sites/NA/Products/CMF/AcellularDermis/Pages/DermaMatrix_Acellular_Dermis.aspx, (6 Feb 2013)
83. lifecell.com, http://www.lifecell.com/health-care-professionals/lifecell-products/allodermr-regenerative-tissue-matrix/allodermr-tissue-matrix-defined/, (6 Feb 2013)
84. wmt.com, http://www.wmt.com/softtissue/graftjacket_right.asp, (6 Feb 2013)
85. ideal-ms.com, http://ideal-ms.com/healthcare-matriderm/, (6 Feb 2013)
86. medline.com, http://www.medline.com/wound-skin-care/matristem/, (6 Feb 2013)
87. woundsource.com, http://www.woundsource.com/product/e-z-derm, (6 Feb 2013)

88. cookmedical.com, http://www.cookmedical.com/feature.do?id=Biodesign, (6 Feb 2013)
89. covidien.com, http://www.covidien.com/hernia/biologics/permacol(6 Feb 2013)
90. davol.com, http://www.davol.com/products/soft-tissue-reconstruction/hernia-repair/biologics/collamend-fm-implants/(6 Feb 2013)
91. davol.com, http://www.davol.com/products/soft-tissue-reconstruction/hernia-repair/biologics/xenmatrix-surgical-graft/(6 Feb 2013)
92. biologiq.nl,http://www.biologiq.nl/UserFiles/Case%20report%20Collagen_Dressing_Clinical_Studies.pdf(6 Feb 2013)
93. lifecell.com, http://www.lifecell.com/health-care-professionals/lifecell-products/stratticetm-reconstructive-tissue-matrix/(6 Feb 2013)
94. mesynthes.com,http://www.mesynthes.com/index.php?view=article&id=7&option=com_content&Itemid=10(6 Feb 2013)
95. synovissurgical.com, www.synovissurgical.com/veritas_collagen_matrix.php(6 Feb 2013)
96. hansbiomed.com, http://www.hansbiomed.com/english/2product/2.3/2.3.3_surederm.htm(6 Feb 2013)
97. davol.com, http://www.davol.com/products/soft-tissue-reconstruction/hernia-repair/biologics/allomax-surgical-graft/(6 Feb 2013)
98. mentorwwllc.com, http://www.mentorwwllc.com/pdf/pids/NEOFORM.pdf(6 Feb 2013)
99. ethicon360.com, http://www.ethicon360.com/products/flex-hd-acelular-hydrated-dermis-family(6 Feb 2013)
100. bioarthro.org, http://bioarthro.org/rotatorcuffpatch_faq.php(6 Feb 2013)
101. bioarthro.org, http://bioarthro.org/tendonwrap.php(6 Feb 2013)

102. cookmedical.com, http://www.cookmedical.com/newsDetail.do?id=63(6 Feb 2013)
103. cookmedical.com, http://www.cookmedical.com/sur/content/mmedia/FP0007-3B.pdf(6 Feb 2013)
104. businesswire.com, http://www.businesswire.com ews/home/20070621005315/en/Pegasus-Biologics- OrthADAPT-TM-Bioimplant-Receives-CE(6 Feb 2013)
105. pentabiomedical.com, http://www.pentabiomedical.com/en/prodotti/bioset-dbm(6 Feb 2013)
106. pentabiomedical.com. http://www.pentabiomedical.com/en/prodotti/sterling-matrice-biologica(6 Feb 2013)
107. depuy.com, http://www.depuy.com/healthcare-professionals/product-details/restore-orthobiologic-soft-tissue-implant(6 Feb 2013)
108. synovissurgical.com, http://www.synovissurgical.com/dura_guard_dural_repair_patch.php(6 Feb 2013)
109. synovissurgical.com, http://www.synovissurgical. com/vascu_guard_peripheral vascular_patch.php(6 Feb 2013)
110. synovissurgical.com, http://www.synovissurgical.com/peri_guard_repair_patch.php(6 Feb 2013)
111. zimmerindia.com, http://www.zimmerindia.com/content/pdf/en-US/Collagen_Repair_Patch_for_Rotator_Cuff_Tendon_Repair.pdf(6 Feb 2013)
112. Jennifer M. Singelyn, Jessica A. DeQuach, Sonya B. Seif-Naraghi, Robert B. Littlefield, Pamela J. Schup-Magoffin, Karen L. Christman, Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering, *Biomaterials* 30 (2009), pp-5409–5416

113. Alejandro Nieponice, Thomas W. Gilbert, and Stephen F. Badylak. Reinforcement of Esophageal Anastomoses Withan Extracellular Matrix Scaffold in a Canine Model. *Ann ThoracSurg*, 82, (2006), pp-2050-2058

114. Anthony Callanan, Niall F. Davis, Michael T. Walsh, Timothy M. McGloughlin. Mechanical characterisation of unidirectional and cross-directionalmultilayered urinary bladder matrix (UBM) scaffolds. *Medical Engineering & Physics* 34 (2012), pp-1368– 1374

115. L.E. Flynn. The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of humanadipose-derived stem cells. *Biomaterials* 31 (2010), pp-4715-4724

116. Basak E Uygun, Alejandro Soto-Gutierrez, Hiroshi Yagi, Maria-Louisa Izamis, MariaA Guzzardi, Carley Shulman, Jack Milwid, Naoya Kobayashi, Arno Tilles, Francois Berthiaume, MartinHertl, Yaakov Nahmias, Martin L Yarmush, and KorkutUygun. Organ reengineering through development of a transplantablerecellularized liver graft using decellularized liver matrix. *Nat Med.* 16(2010), pp-814–820.

117. Bryan N. Brown, Christopher A. Barnes, Rena T. Kasick, Roger Michel, Thomas W. Gilbert,Donna Beer-Stolz, David G. Castner, Buddy D. Ratner, Stephen F. Badylak. Surface characterization of extracellular matrix scaffolds. *Biomaterials* 31 (2010), pp-428–437

118. Donald O. Freytes, Stephen F. Badylak, Thomas J. Webster, Leslie A. Geddes, Ann E. Rundell. Biaxial strength of multilaminated extracellular matrix scaffolds. *Biomaterials* 25 (2004), pp-2353–2361

119. Thomas W. Gilbert, John Freund, and Stephen F. Badylak. Quantification of DNA in Biologic Scaffold Materials. *J Surg Res.* , 152(2009), pp-135–139

120. Bryan N. Brown, John M. Freund, Li Han, J . Peter Rubin, Janet E. Reing, Eric M. Jeffries, Mathew T. Wolf, Stephen Tottey,Christopher A. Barnes, Buddy D. Ratner, Stephen F. Badylak. Comparison of Three Methods for the Derivationof a Biologic Scaffold Composed of AdiposeTissue Extracellular Matrix. *Tissue Engineering: Part C* 17, (2011),pp-411-421

121. D. Adam Young, Dina O. Ibrahim, Diane Hu, Karen L. Christman. Injectable hydrogel scaffold from decellularized human lipoaspirate. *Acta Biomaterialia* 7 (2011) pp-1040–1049

122. Ji Suk Choi,BeobSoo Kim, Jun Young Kim,Jae Dong Kim, Young Chan Choi,Hyun - Jin Yang, Kinam Park,Hee Young Lee,Yong Woo Cho. Decellularized extracellular matrix derived from human adipose tissueas a potential scaffold for allograft tissue engineering. *Journal of biomedical materials research A*, 97, (2011), pp-292-299

123. Ji Suk Choi, Hyun-Jin Yang, BeobSoo Kim, Jae Dong Kim, Jun Young Kim, BongyoungYoo,Kinam Park, Hee Young Lee, Yong Woo Cho. Human extracellular matrix (ECM) powders for injectable cell delivery and adiposetissue engineering. *Journal of Cont rolled Release* 139 (2009), pp- 2–7

124. Matthew T. Wolf, Kerry A . Daly, Janet E. Reing, Stephen F. Badylak. Biologic scaffold composed of skeletal muscle extracellular matrix. *Biomaterials* 33 (2012) , pp-2916-2925

125. Matthew T. Wolf, Kerry A . Daly, Ellen P. Brennan-Pierce, Scott A . Johnson, Christopher A. Carruthers , Antonio D' Amore a, Shailesh P. Nagarkar , Sachin S. Velankar,Stephen F. Badylak. A hydrogel de rived from decellularize d dermal extracellular matrix. *Biomaterials* 33 (2012), pp- 7028- 7038

126. Peter M. Crapo, Christopher J. Medberry, Janet E. Reing, Stephen Tottey, Yolandi van der Merwe,Kristen E. Jones, Stephen F. Badylak. Biologic scaffolds composed of central nervous system extracellular matrix, *Biomaterials* 33 (2012), pp- 3539-3547

127. Qiang Yang, Jiang Peng, QuanyiGuo, Jingxiang Huang, Li Zhang, Jun Yao,Fei Yang, Shenguo Wang, WenjingXu, Aiyuan Wang, Shibi Lu. A cartilage ECM-derived 3-D porous acellular matrix scaffoldfor in vivo cartilage tissue engineering with PKH26-labeledchondrogenic bone marrow-derived mesenchymal stem cells. *Biomaterials* 29 (2008), pp- 2378-2387

128. Thomas W. Gilbert, Sebastien Gilbert, Matthew Madden, Susan D. Reynolds, Stephen F. Badylak. Morphologic Assessment of Extracellular MatrixScaffolds for Patch Tracheoplasty in a CanineModel. *Ann ThoracSurg*, 86, (2008), pp-967-974

129. Birzabith Mendoza-Novelo , Eva E. Avila , Juan V. Cauich-Rodríguez , Eduardo Jorge-Herrero. Decellularization of pericardial tissue and its impact on tensile viscoelasticity and glycosaminoglycan content, *Acta Biomaterialia* 7 (2011), pp-1241–1248

130. Donald O. Freytes, Stephen F. Badylak, Thomas J. Webster, Leslie A. Geddes, Ann E. Rundell. Biaxial strength of multilaminated extracellular matrix scaffolds. *Biomaterials* 25 (2004), pp- 2353–2361

131. Bryan N. Brown, Christopher A. Barnes, Rena T. Kasick, Roger Michel, Thomas W. Gilbert,Donna Beer-Stolz, David G. Castner, Buddy D. Ratner, Stephen F. Badylak. Surface characterization of extracellular matrix scaffolds. *Biomaterials* 31 (2010), pp-428–437

132. Webster TJ, Siegel RW, Bizios R. Enhanced surface and mechanical properties of nano phase ceramics to achieve orthopaedic/dental implant efficacy. *Key Engineering Materials* 92,(2001), pp- 321-4.

133. Webster TJ, Ergun C, Doremus RH, Siegel RW, Bizios R. Enhanced functions of osteoblasts on nanophase ceramics. *Biomaterials* 21(2000), pp- 1803-10.

134. Webster TJ. Specific proteins mediate enhanced osteoblast adhesion on nanophase ceramics. *J Biomed Mater Res,* 51, (2000), pp- 475-83.

135. [128] Zhu W, Zhang X, Wang D, Lu W, Ou Y, Han Y. Experimental study on the conduction function of nano-hydroxyapatite artificial bone. *Micro Nano Lett, IET* , 5, (2010), pp-19–27.

136. [129] Wang X, Li Y, Wei J, de Groot K. Development of biomimetic nanohydroxyapatite/poly (hexamethylene adipamide) composites. *Biomaterials,* 23, (2002), pp-4787–91.

137. [130] Gutwein LG, Webster TJ. Increased viable osteoblast density in the presence of nanophase compared to conventional alumina and titanium particles. *Biomaterials,* 25, (2004), pp-4175–83.

138. [131] Wahl DA, Czernuszka JT. Collagen-hydroxyapatite composites for hard tissue repair. *Eur Cell Mater,* 11, (2006),pp-43–56.

139. Sopyan I, Mel M, Ramesh S, Khalid KA. Porous hydroxyapatite for artificial bone applications. *Sci Technol Adv Mater,* 8, (2007), pp-116–23.

140. Kim YG, Seo DS, Lee JK. Comparison of dissolution resistance in artificial hydroxyapatite and biologically derived hydroxyapatite ceramics. *J Phys Chem Solids*,69, (2008),pp-1556–9.

141. Wang D, Han Y, Zhu W, Lu W, Liu H, Jiang H, Nano-hydroxyapatite artificial bone with different pore sizes to repair radial defect in rabbits. *Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu (J Clin Rehab Tissue Eng Res),*11, (2007),pp-9641–5.

142. Lim BK, Sun F, Ryu SC, Koh K, Han DW, Lee J. Hydroxyapatite coatingon damaged tooth surfaces by immersion. *Biomed Mater,* 4, (2009),pp-025017.

143. Ryu SC, Lim BK, Sun F, Koh K, Han DW, Lee J. Regeneration of a microscratched tooth enamel layer by nanoscale hydroxyapatite solution. *Bull Korean Chem Soc*, 30, (2009), pp-887.

144. Jiehong Liao, Xuan Guo, K. Jane Grande-Allen, F. Kurtis Kasper, Antonios G. Mikos. Bioactive polymer/extracellular matrix scaffolds fabricated with a flow perfusion bioreactor for cartilage tissue engineering. *Biomaterials* 31 (2010), pp-8911-8920

145. Hongxu Lu, Takashi Hoshiba, Naoki Kawazoe, Guoping Chen. Autologous extracellular matrix scaffolds for tissue engineering. *Biomaterials* 32 (2011), pp-2489-2499

146. Matthew T. Wolf, Kerry A . Daly, Janet E. Reing, Stephen F. Badylak. Biologic scaffold composed of skeletal muscle extracellular matrix. *Biomaterials* 33 (2012) , pp-2916-2925

147. Peter M. Crapo, Christopher J. Medberry, Janet E. Reing, Stephen Tottey, Yolandi van der Merwe, Kristen E. Jones, Stephen F. Badylak. Biologic scaffolds composed of central nervous system extracellular matrix, *Biomaterials* 33 (2012), pp- 3539-3547

148. Jiehong Liao, Xuan Guo, Dan Nelson, F. Kurtis Kasper, Antonios G. Mikos. Modulation of osteogenic properties of biodegradable polymer/extracellular matrix scaffolds generated with a flow perfusion bioreactor. *Acta Biomaterialia* 6 (2010) , pp-2386–2393

149. Shuaijun Jia, Lie Liu, Weimin Pan, Guolin Meng, Chunguang Duan, Laquan Zhang, Zhuo Xiong, and Jian Liu. Oriented cartilage extracellular matrix-derived scaffold for cartilage tissue engineering. Journal of Bioscience and Bioengineering. 5, (2012), pp-647 – 653

150. Qiang Yang, Jiang Peng, Quanyi Guo, Jingxiang Huang, Li Zhang, Jun Yao, Fei Yang, Shenguo Wang, Wenjing Xu, Aiyuan Wang, Shibi Lu. A cartilage ECM-derived 3-D porous acellular matrix scaffold for in vivo cartilage tissue engineering with PKH26-labeled chondrogenic bone marrow-derived mesenchymal stem cells. *Biomaterials* 29 (2008),pp- 2378-2387

151. Anthony Callanan, Niall F. Davis, Michael T. Walsh, Timothy M. McGloughlin. Mechanical characterisation of unidirectional and cross-directional multilayered urinary bladder matrix (UBM) scaffolds. *Medical Engineering & Physics* 34 (2012), pp-1368– 1374

152. N.F. Davis, A. Callanan, B.B. McGuire, R. Mooney, H.D. Flood, T.M. McGloughlin. Porcine extracellular matrix scaffolds in reconstructive urology: An e x vivo comparative study of their biomechanical properties. *Journal of Mechanical behavior of Biomedical Materials* 4(2011), pp-375-382

153. Thomas W. Gilbert, PhD, Sebastien Gilbert, MD, Matthew Madden, BS, Susan D. Reynolds, PhD, Stephen F. Badylak, DVM, MD. Morphologic Assessment of Extracellular Matrix Scaffolds for Patch Tracheoplasty in a Canine Model. *Ann Thorac Surg* 86, (2008), pp- 967-974

154. Aidan J. Cloonan, Michael R. O'Donnell, William T. Lee, Michael T. Walsh, Eamonn DeBarra, Tim M. McGloughlin. Spherical Indentation of free-standing acellular Extracellular Matrix Membranes

155. Alejandro Nieponice, Thomas W. Gilbert, and Stephen F. Badylak. Reinforcement of Esophageal Anastomoses With an Extracellular Matrix Scaffold in a Canine Model. *Ann Thorac Surg*, 82, (2006), pp-2050-2058

156. Cheng Zhe Jin,Byung Hyune Choi,So Ra Park,Byoung- Hyun Min. Cartilage engineering using cell-derived extracellular matrix scaffold in vitro. *Journal of Biomedical Materials Research Part A* 15(2010), pp-1567-77

157. Ji Suk Choi, Beob Soo Kim, Jun Young Kim,Jae Dong Kim, Young Chan Choi,Hyun -Jin Yang, Kinam Park, Hee Young Lee,Yong Woo Cho. Decellularized extracellular matrix derived from human adipose tissue as a potential scaffold for allograft tissue engineering. *Journal of biomedical materials research A* , 97 A, (2011), pp-292-299

158. Jason Hodde, Abram Janis · David Ernst ·David Zopf · Debra Sherman · Chad Johnson. Effects of sterilization on an extracellular matrix scaffold: Part I. Composition and matrix architecture. *J Mater Sci: Mater Med* 18, (2007) , pp-537–543

159. Jason Hodde · Abram Janis · Michael Hiles. Effects of sterilization on an extracellular matrix scaffold:Part II. Bioactivity and matrix interaction. *J Mater Sci: Mater Med* 18, (2007) , pp-545–550

160. Stephen Badylak, Steven Arnoczky, Pam Plouhar, Roger Haut, Vince Mendenhall, Rhonda Clarke, and Christopher Horvath. Naturally Occurring Extracellular Matrix as a Scaffold for Musculoskeletal Repair. Clinical orthopaedics and related research Number 3678, (1999),pp- s333-s342

161. Stephen F. Badylak, Paul V. Kochupura, Ira S. Cohen, Sergey V. Doronin, Adam E. Saltman, Thomas W. Gilbert, Damon J. Kelly, Ronald A. Ignotz, and Glenn R. Gaudette. The Use of Extracellular Matrix as an Inductive Scaffold for the Partial Replacement of Functional Myocardium. *Cell Transplantation*, 15, (2006), pp. S29 – S40

162. Ji Suk Choi, Hyun-Jin Yang, Beob Soo Kim, Jae Dong Kim, Jun Young Kim, Bongyoung Yoo, Kinam Park, Hee Young Lee, Yong Woo Cho. Human extracellular

matrix (ECM) powders for injectable cell delivery and adipose tissue engineering. *Journal of Cont rolled Release* 139 (2009),pp- 2– 7

163. A.V. Piterina, L.M. Davis, C.L. Meaney, A.J. Cloonan, M.T. Walsh and Prof. Tim.M. McGloughlin. Cell-Seeded Decellularised Extracellular Matrices As An Advanced Approach For Tissue Engineering. Eccomas – International Conference On Tissue Engineering 2009

164. Denis E Solomon. An in vitro examination of an extracellular matrix scaffold for use in wound healing. *Int J Exp.Path*, 83, (2002) ,pp- 209-216.

165. Keith A. Robinson, Jinshen Li, Megumi Mathison, Alka Redkar, Jianhua Cui, Nicolas A.F, Chronos, Robert G. Matheny and Stephen F. Badylak. Extracellular Matrix *Scaffold for Cardiac Repair*. 112, (2005), pp- I-135-I-143.

166. Vineet Agrawal, Bryan N. Brown, Allison J. Beattie, Thomas W. Gilbert, and Stephen F. Badylak. Evidence of Innervation following Extracellular Matrix Scaffold Mediated Remodeling of Muscular Tissues. *J Tissue Eng Regen Med*. 3(2009), pp-590–600

167. L.E. Flynn. The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells. *Biomaterials* 31 (2010),pp- 4715-4724

168. Allison E.B. Turner, Claire Yu, Juares Bianco, John F. Watkins, Lauren E. Flynn. The performance of decellularized adipose tissue microcarriers as an inductive substrate for human adipose-derived stem cells. *Biomaterials* 33 (2012), pp-4490-4499

169. Beob Soo Kim & Ji Suk Choi & Jae Dong Kim & Young Chan Choi & Yong Woo Cho. Recellularization of decellularized human adipose-tissue-derived extracellular matrix sheets with other human cell types. *Cell Tissue Res,* , 348(2012),pp-559-67

170. D. Adam Young, Dina O. Ibrahim, Diane Hu, Karen L. Christman. Injectable hydrogel scaffold from decellularized human lipoaspirate. *Acta Biomaterialia* 7 (2011), pp-1040–1049

171. Ji Suk Choi, Beob Soo Kim, Jun Young Kim, Jae Dong Kim, Young Chan Choi, Hyun -Jin Yang, Kinam Park, Hee Young Lee, Yong Woo Cho. Decellularized extracellular matrix derived from human adipose tissue as a potential scaffold for allograft tissue engineering. *Journal Of Biomedical Materials Research A* , 97A,(2011), pp- 292-299

172. Micha el P. Franci s, Patrick C. Sachs, Parthasarathy A. Madurantakam,Scott A. Sell, Lynne W. Elmore ,Gary L. Bo wlin,Shawn E. Holt. Electrospinning adipose tissue-derived extracellular matrix for adipose stem cell culture. *Journal Of Biomedical Materials Research* 00A, (2012), pp-1-9

173. Allison Eugenia Bogart Turner(2010). Matrix-Derived Microcarriers For Adipose Tissue Engineering(Master's thesis) Queen's University, **http://hdl.handle.net/1974/6214**

174. Stephen F. Badylak, Kinam Park, Nicholas Peppas, George McCabe, and Mervin Yoder. Marrow-derived cells populate scaffolds composed of xenogeneic extracellular matrix. *Experimental Hematology* 29 (2001),pp- 1310–1318

175. David M. Hoganson, Elisabeth M. O' Doherty, Gwen E. Owens, Dina O. Harilal, Scott M. Goldman, Chris M. Bowley, Craig M. Neville, Russell T. Kronengold, Joseph P. Vacanti. The retention of extracellular matrix proteins and angiogenic and mitogenic cytokines in a decellularized porcine dermis. *Biomaterials* 31 (2010),pp-6730-6737

176. Thomas W. Gilbert, Donna Beer Stolz, Frank Biancaniello, Abby Simmons-Byrd, Stephen F. Badylak. Production and characterization of ECM powder: implications for tissue engineering applications. *Biomaterials* 26 (2005), pp-1431–1435

177. Thomas W. Gilbert, John Freund, and Stephen F. Badylak. Quantification of DNA in Biologic Scaffold Materials. *J Surg Res*.,152(2009),pp-135–139